Remarks

Claims 44-47 were pending. By this amendment, no claims are cancelled or added. Therefore, claims 44-47 are still pending.

Claims 44, 46, and 47 are amended to clarify the claims.

The specification is amended to recite the parent number of the parent application, and to amend the abstract in view of the claims being prosecuted.

No new matter is introduced by this amendment, and no amendments are made to distinguish prior art.

Summary of Telephone Interview with the Examiner

Applicants thank Examiner Para for the courtesy of a telephone interview with Applicant's Representative Sheree Lynn Rybak, Ph.D. on March 1, 2007. During this interview the outstanding 35 U.S.C. §§ 112, first paragraph and 102(b) rejections were discussed. Agreement was not reached.

Examiner Para explained that the claims were rejected under 35 U.S.C. § 112, first paragraph, because the specification does not describe how to isolate the endophytes, and no deposit of endophytes has been made. Applicant's representative explained that methods of isolating endophytes are routine, and thus there is no duty to make such a disclosure in the specification. In addition, Applicant's representative noted that Applicant would be willing to make a deposit of endophyte as it is Applicant's understanding that such a deposit would not constitute new matter (see MPEP 2406.02), if this would overcome the outstanding rejection. Examiner Para recommended filing a CIP application.

With regards to the 35 U.S.C. § 102(b) rejection, Applicant's representative noted that the units in the cited Siegel *et al.* document are in ppm (parts per million), not ppb (parts per billion). The claims recite ppb, not ppm. Therefore, Siegel *et al.* does not anticipate the claims. Examiner Para agreed to reconsider Siegel *et al.* in view of this information.

Objections to the Specification

The abstract was objected to on the ground that it is not drawn to the claimed invention. The abstract has been amended to clarify that the application is directed to endophytes.

The specification was objected to on the ground that page 14 of the specification recites that the seeds had about 183 ppb of ergovaline, while claim 47 recited about 55 ppb of ergovaline. Claim 47 has been amended to clarify that the level of ergovaline in seeds is about 183 ppb.

In view of these amendments, Applicant requests that the objections to the specification be withdrawn.

35 U.S.C. § 101

Claims 44-47 are rejected as being directed to non-statutory subject matter. Applicant requests reconsideration.

Claims 44 and 46 are amended to recite that the endophytes are isolated.

In view of this amendment, Applicant requests that the 35 U.S.C. § 101 rejection be withdrawn.

35 U.S.C. § 112, written description

Claims 44-47 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Applicant disagrees and requests reconsideration.

It is asserted in the Office action that the claims are drawn to any endophyte of a tall fescue. This is incorrect. The claims are directed to endophytes isolated from a tall fescue grasses grown from a particular ATCC seed deposit (ATCC PTA-3825) (claims 44-45) or endophytes isolated from the deposited seed itself (claims 46-47).

It is asserted the Applicant did not isolate the claimed endophyte. It is not relevant whether the inventor actually reduced to practice the claimed invention (for example by isolating endophytes from the plant or seed), or constructively reduced to practice (for example by providing sufficient information for one skilled in the art to reduce to practice the claimed invention) (see MPEP 2406.02).

Applicant disclosed the seed from which the endophyte can be isolated or which can be used to grow grass from which endophytes can be isolated. Methods of isolating endophytes from seed or grass plants were routine in the art as of the priority date of the present application

(January 30, 2002). For example, Bony *et al.* (*New Phytologist* 152:125-37, 2001; Exhibit A), describe a method of isolating endophytes from germinated grass seed (see page 126, last two paragraphs). Siegel *et al.* (*Annu. Rev. Phytopathol.* 25:293-315, 1987; Exhibit B) describe a method of isolating endophytes from grass plants (see pages 297-298), as do Latch *et al.* (*Mycotaxon*, XX:535-50, 1984; Exhibit C, see page 536). As stated in MPEP 2164.05(a), "The specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public." Therefore, as the seed from which the endophyte can be isolated (or can be used to grow a plant from which the endophyte can be isolated) is provided under the terms of the Budapest Treaty, and methods for isolating endophytes are well-known to those skilled in the art, the specification provides ample written description for the claimed isolated endophytes.

Therefore, Applicant requests that the 35 U.S.C. § 112, first paragraph written description rejection be withdrawn.

35 U.S.C. § 112, enablement

Claims 44-47 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Applicant disagrees and requests reconsideration.

Although the claimed endophytes have not yet been deposited under the terms of the Budapest Treaty, seeds from which the endophytes can be isolated are deposited under the terms of the Budapest Treaty (ATCC PTA-3825). As described above, one skilled in the art can isolate endophyte from such seed using routine methods. Therefore, the claims are enabled, even in the absence of a biological deposit of the endophytes themselves.

However, Applicant is willing to make a deposit of the claimed endophyte if this would expedite prosecution. Unfortunately, Examiner Para stated during the telephone interview with Applicant's representative that making a biological deposit of the claimed endophyte at this time would constitute new matter. Applicant disagrees and requests reconsideration in view of MPEP 2406.02, which states "the rules will not preclude such a situation as there is no requirement in the patent law that an actual reduction to practice occur as a condition precedent to filing a patent application." This section of the MPEP states that it is acceptable to make an original deposit after the effective filing date of an application for patent. In such a case, "applicant is required to promptly submit a statement from a person in a position to corroborate that the biological

material which is deposited is a biological material specifically identified in the application (the filing date of which is relied upon) as filed." Applicant is willing to make such a statement in combination with a biological deposit to expedite prosecution.

If the Office agrees with Applicant's argument, Applicant is willing to make a deposit under the terms of the Budapest Treaty and make the statement required under MPEP 2406.02, and will amend the specification accordingly.

Therefore, Applicant requests that the 35 U.S.C. § 112, first paragraph enablement rejection be withdrawn.

35 U.S.C. § 102(b)

Claims 44-47 are rejected under 35 U.S.C. § 102(b) as anticipated by Siegel *et al.* (*J. Chem. Ecol.* 16:3301, 1990). Applicant disagrees and requests reconsideration.

It is asserted on page 7 of the Office action that Siegel *et al.* teach a tall fescue having endophyte and 44 ppb of lolitrem B and 1.3 ppb of ergovaline (Table 2, plant 12), and thus anticipate the claims. This assertion is incorrect, as the units in Table 2 are ppm (parts per million) not ppb (parts per billion). Therefore, plant 12 disclosed in Siegel *et al.* has significantly more lolitrem B and ergovaline than the claimed endophytes.

Because Siegel *et al.* do not anticipate the claims, Applicant requests that the 35 U.S.C. § 102(b) rejection be withdrawn.

If there are any minor issues to be resolved before a Notice of Allowance is issued, the Examiner is invited to telephone the undersigned.

Respectfully submitted,

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New Phytologist

Research

The relationship between mycotoxin synthesis and isolate morphology in fungal endophytes of *Lolium perenne*

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Summary

- Variability in the fungal endophytes of 83 natural populations of *Lolium perenne* (perennial ryegrass) from Europe was assessed.
- One plant per population was used for endophyte isolation and mycotoxin analysis. Variability in three isozymes, colony morphology and growth rate on potato dextrose agar (PDA), and synthesis of ergovaline, lolitrem B and peramine was recorded.
- Three species were found among 94 strains isolated: *Neotyphodium lolii*, *Neotyphodium* sp. (LpTG-2) and *Gliocladium*-like. The most frequent species was *N. lolii*, which showed high variability. In 12 populations, a single plant harboured two different endophytes. One-third of the isolates of *N. lolii* did not produce ergovaline whereas a few isolates did not produce lolitrem B. Ergovaline and lolitrem B-deficient strains, but not the few peramine-deficient isolates, had characteristic morphologies on PDA. No isolate was deficient for both ergovaline and lolitrem B synthesis.
- Selection of ergovaline and lolitrem-deficient strains based only on the morphology of the isolates in culture may be possible.

Key words: fungal endophytes, *Lolium perenne* (perennial ryegrass), *Neotyphodium*, intraspecific fungal variability, ergovaline, lolitrem B, peramine.

Introduction

The presence of fungal endophytes in the seeds of *Lolium perenne* L. was first observed by Guérin (1898). McLennan (1920) studied the distribution and development of the mycelium in the different organs of the grass. Sampson (1937) was the first to isolate an endophytic fungus from *L. perenne* and to grow it in pure culture.

In New Zealand, a damaging tremorgenic disease of sheep and cattle, 'ryegrass staggers', had long been reported and attributed to the consumption of perennial ryegrass by grazing animals (Cunningham & Hartley, 1959). Later on, ryegrass staggers was shown to be caused by alkaloids (Aasen *et al.*, 1969) and the implication of the fungal endophyte in the toxicosis was eventually established by Fletcher & Harvey (1981).

Sampson (1933) observed the presence of two different endophytic fungi in *Lolium perenne* and Latch *et al.* (1984) isolated them. The first type was thin, poorly stainable and highly branched. It was generally sterile in culture, but the production of a few conidiophores resembling *Gliocladium* led to this species being called '*Gliocladium*-like' (Philipson, 1989).

The second, more frequent, type consisted of twisted, poorly branched hyphae. It was shown to belong to clavicipitaceae and described as a new species, *Acremonium loliae* Latch, Christensen and Samuels (later changed to *Acremonium lolii*). Within the genus *Acremonium*, this species was classified in a section *Albo-lanosa* created by Morgan-Jones & Gams (1982) to include the anamorphs of the clavicipitaceae. However, the fungi belonging to this section were reclassified by Glenn *et al.* (1996) into the new genus *Neotyphodium*, the clavicipitaceous endophytes of *L. perenne* being named *N. lolii* (Latch, Christensen & Samuels) Glenn, Bacon & Hanlin.

Christensen *et al.* (1991, 1993) introduced an additional distinction among the clavicipitaceous endophytes of perennial

ryegrass on the basis of growth rate, isolation delay, sporulation at 23°C and isozyme pattern. The majority of isolates could be accommodated as *Neotyphodium lolii*. However, a few isolates showing higher growth were just named by the abbreviation LpTG-2.

The clavicipitaceous endophytes of perennial ryegrass are known to synthesize several mycotoxins, among which three are particularly important: lolitrem B, a tremorgenic molecule responsible for 'ryegrass staggers'; ergovaline, a compound belonging to the ergopeptine group, which has vasoconstrictive effects and causes various diseases on grazing mammals ('fescue-foot' and 'fescue toxicosis'); and peramine, a tripeptide which is repellent and toxic for insects but not for mammals. The strains of LpTG-2 which have been studied synthesized ergovaline and peramine, but not lolitrem B, while variation was observed among strains of *N. lolii* for the synthesis of lolitrem B, ergovaline and peramine (Christensen *et al.*, 1993). Schardl *et al.* (1994) showed that LpTG-2 is a heteroploid species originating from interspecific hybridization between *Neotyphodium lolii* and the parasitic species *Epichloë typhina*.

The dual action of clavicipitaceous endophytes (beneficial effects on the host vs risk of toxicity for grazing animals) sets forage grass breeders special problems. Sometimes, these have been solved by removing the endophyte from the cultivars selected for pasture and by maintaining or introducing it into varieties selected for turf. Another strategy, developed mainly in New Zealand (Latch, 1989), proposed to select harmless clavicipitaceous endophytes (producing no or little ergovaline or lolitrem) and to inoculate these strains to commercial cultivars. This strategy involves an extensive study of the variability of the endophytes of *Lolium perenne*. Such research was conducted on a large scale in New Zealand (Latch, 1994; Fletcher & Easton, 1997).

Very few studies have been conducted on this subject in Europe, despite the fact that *Lolium perenne* probably originated from the Near East and has diversified in Europe (Balfourier *et al.*, 2000). Maximum genetic variability would therefore be expected in Europe.

In France, 547 natural populations of *Lolium perenne* were collected from 1983 to 84 (Charmet *et al.*, 1990, 1993; Balfourier & Charmet, 1991). About half of these populations were sampled at random and checked for the presence of endophytes (Lewis *et al.*, 1997; Ravel, 1997). One, or several, endophytes were found in 188 populations out of 262 (72%). The present study was conducted on part of this material. The objectives were to identify the fungal species involved and to describe intraspecific variability, particularly as concerns the production of mycotoxins.

Materials and Methods

Material

Seventy-three populations with endophytes were selected from the populations studied by Ravel (1997). They were conserved at INRA Clermont-Ferrand as seeds and/or living plants (Table 1a). The material was selected on geographical and ecological bases to achieve homogeneous cover of the country, and to include a wide range of ecological situations.

For comparison, 10 populations of European, nonFrench origin, were included in the study (Table 1b). In addition, six standard isolates (four of *Neotyphodium lolii* and two of species LpTG-2) were obtained from G. C. M. Latch (from AgResearch, New Zealand (Table 1c). These last isolates originated from natural populations in Spain and Southern France.

Methods

General procedure

For each population, one plant had been selected in the field, and the presence of an endophyte had been verified. The 83 populations were represented by seeds originating from only one plant per population.

Isolations were carried out from the 83 seed lots. All the isolates were grown on PDA (Potato Dextrose Agar, Sigma Chemical, 39 g l^{-1}) to observe their macroscopical morphology and measure their linear growth. Mycelium grown in liquid culture was checked for the isoforms of three enzymes, to allow the taxonomical identification of the isolates.

Straws from the 83 plants from which the seeds had been taken had been harvested in early August, frozen and lyophilized, and the analysis of ergovaline, lolitrem B and peramine was carried out on this material.

Isolation

Seeds were sterilized for 4 h in commercial bleach (12°Cl.), then rinsed with sterile water and sterilized again for 3 min in Bayrochlor® (from Bayrochlor Company). They were rinsed three times with sterile water and the glumellas were removed. The seeds were air-dried and then sown in Petri dishes containing PDA plus penicillin and streptomycin (100 µg g^{-1} each). Approximately 10 seeds were placed in each dish. The Petri dishes were sealed with adhesive tape, placed on their side and incubated in the dark at 23°C, the seeds, slightly sunk in agar, were aligned horizontally, with their coleoptiles upwards (Fig. 1).

Every 2 d the seeds were observed for germination and the appearance of mycelium on the medium. Under these conditions, the fungus was observed to grow from the cotyledon, the coleoptile and even the seminal roots. In the majority of cases, the coleoptile was cut into pieces with a microscalpel and the pieces subcultured on a new PDA medium. The fungi obtained were subcultured, paying particular attention to the appearance of any morphologically different colonies from the same seed lot: colonies showing a clearly different aspect or growth rate were subcultured separately.

Table 1 Taxonomical identification, morphology and growth rate of the isolates. (a) Populations of French origin (b) Populations of non-French origin (c) Reference isolates

(a) n° popul	Isolates	Fungal species[1]	MG[2]	Linear growth[3]
10119	10119	*Neotyphodium lolii*	III	C7
10151	10151	*N. lolii*	III	C6
10156	10156	*N. lolii*	III	C6
10170	10170	*N. lolii*	III	C6
10180	10180	*N. lolii*	II	C7
10184	10184	*N. lolii*	III	C4
10208	10208	*N. lolii*	I	C9
10224	10224	*N. lolii*	I	C9
10251	10251	*N. lolii*	II	C7
10256	10256 A	*N. lolii*	III	C6
	10256 B	*N. lolii*	IV	C2
10261	10261	*N. lolii*	III	C5
10266	10266	*N. lolii*	II	C4
10304	10304	*N. lolii*	III	C6
10319	10319	*N. lolii*	III	C6
10359	10359	*N. lolii*	III	C6
10361	10361 A	*N. lolii*	VI	C7
	10361 B	*N. lolii*	II	C8
10363	10363	***Gliocladium*-like**	VIII	C4
10364	10364	*N. lolii*	II	C7
10365	10365	*N. lolii*	II	C6
10367	10367	*N. lolii*	II	C8
10370	10370 A	*N. lolii*	II	C8
	10370 B	*N. lolii*	III	C6
10405	10405	*N. lolii*	II	C7
10452	10452	*N. lolii*	III	C6
10458	10458	*N. lolii*	III	C5
10505	10505	*N. lolii*	II	C6
10509	10509 A	*N. lolii*	II	C6
	10509 B	*N. lolii*	VI	C5
10515	10515 A	*N. lolii*	V	C2
	10515 B	*N. lolii*	II	C7
10520	10520	*N. lolii*	II	C6
10551	10551	*N. lolii*	III	C6
10557	10557	*N. lolii*	II	C7
10573	10573	*N. lolii*	III	C6
10606	10606	*N. lolii*	II	C6
10611	10611	*N. lolii*	III	C6
10621	10621	*N. lolii*	II	C6
10626	10626	*N. lolii*	II	C7
10656	10656	*N. lolii*	III	C4
10667	10667	*N. lolii*	II	C6
10674	10674	*N. lolii*	III	C7
10675	10675	*N. lolii*	III	C7
10702	10702	*N. lolii*	III	C7
10707	10707	*N. lolii*	III	C5
10710	10710	*N. lolii*	III	C6
10716	10716	*N. lolii*	III	C7
10753	10753	*N. lolii*	III	C6
10763	10763	*N. lolii*	I	C9
10769	10769	*N. lolii*	III	C6
10770	10770	*N. lolii*	III	C5
10873	10873	*N. lolii*	I	C9
10880	10880	*N. lolii*	II	C7
10905	10905	*N. lolii*	I	C9
10906	10906	*N. lolii*	II	C9
10910	10910	*N. lolii*	III	C6
10917	10917	*N. lolii*	III	C6
10954	10954	*N. lolii*	II	C8
10963	10963 A	*N. lolii*	IV	C1
	10963 B	*N. lolii*	V	C4

Table 1 *continued*

(a)

n° popul	Isolates	Fungal species[1]	MG[2]	Linear growth[3]
10972	10972	*N. lolii*	III	C5
11104	11104	*N. lolii*	II	C7
11110	11110	***Gliocladium*-like**	VIII	C5
11115	11115	*N. lolii*	II	C8
11118	11118 A	*N. lolii*	III	C6
	11118 B	*N. lolii*	VI	C7
11151	11151	*N. lolii*	III	C7
11154	11154 A	*N. lolii*	III	C5
	11154 B	***Gliocladium*-like**	VIII	C4
11163	failure of isolation			
11178	11178	*N. lolii*	III	C9
11205	11205 A	*N. lolii*	III	C6
	11205 B	*N. lolii*	VI	C9
11214	11214	*N. lolii*	III	C5
11264	11264	*N. lolii*	III	C7
11274	11274	*N. lolii*	II	C6
11280	11280	*N. lolii*	III	C4
11302	11302	*N. lolii*	III	C1
11314	11314 A	***LpTG-2***	VII	C4
	11314 B	*N. lolii*	III	C6
11315	11315	***LpTG-2***	VII	C4
11442	11442 A	***LpTG-2***	VII	C3
	11442 B	*N. lolii*	IV	C5

(b)

n° popul.	Country	Isolates	Fungal species[1]	MG[2]	Linear growth[3]
20088	Spain	20088	**LpTG-2**	VII	C7
20118	Spain	20118	**LpTG-2**	VII	C4
40128	Italy	40128	**LpTG-2**	VII	C3
40137	Italy	40137 A	**LpTG-2**	VII	C4
		40137 B	*N. lolii*	IV	C2
70036	Poland	70036	*N. lolii*	II	C6
110005	Yugoslavia	110005	*N. lolii*	V	C2
110009	Yugoslavia	110009	*N. lolii*	II	C6
120027	Germany	120027	***Gliocladium*-like**	VIII	C2
130003	Greece	130003	*N. lolii*	IV	C1
210027	Bulgaria	210027	*N. lolii*	V	C3

(c)

Isolates	Fungal species[1]	MG[2]	Linear growth[3]
R 1	**LpTG-2**	VII	C2
R 2	**LpTG-2**	VII	C3
R 3	*N. lolii*	VI	C9
R 4	*N. lolii*	V	C4
R 5	*N. lolii*	V	C3
R 6	*N. lolii*	I	C8

[1]Identification from patterns Malate Dehydrogenase (MDH), Phosphoglucomutase (PGM) and Phosphoglucose Isomerase (PGI). [2]Morphological Groups (Fig. 2). [3]Nonoverlapping groups of growth. Groups are ordered from C1, the group characterized by the highest linear growth, to C9, the group characterized by the lowest linear growth. The average growth in mm d^{-1} is 0.353 for C1, 0.304 for C2, 0.269 for C3, 0.231 for C4, 0.193 for C5, 0.150 for C6, 0.113 for C7, 0.077 for C8 and 0.019 for C9.

Species identification by isozyme analysis

An unambiguous identification of the isolates was obtained by analysis of three isozymes: Malate Dehydrogenase (MDH), Phosphoglucose Isomerase (PGI) and Phosphoglucomutase (PGM). The isolates were grown in V8 liquid medium containing for 1 l: V8 juice (a commercial product constituted of extracts of 8 different vegetables): 100 ml; D-glucose: 10 g;

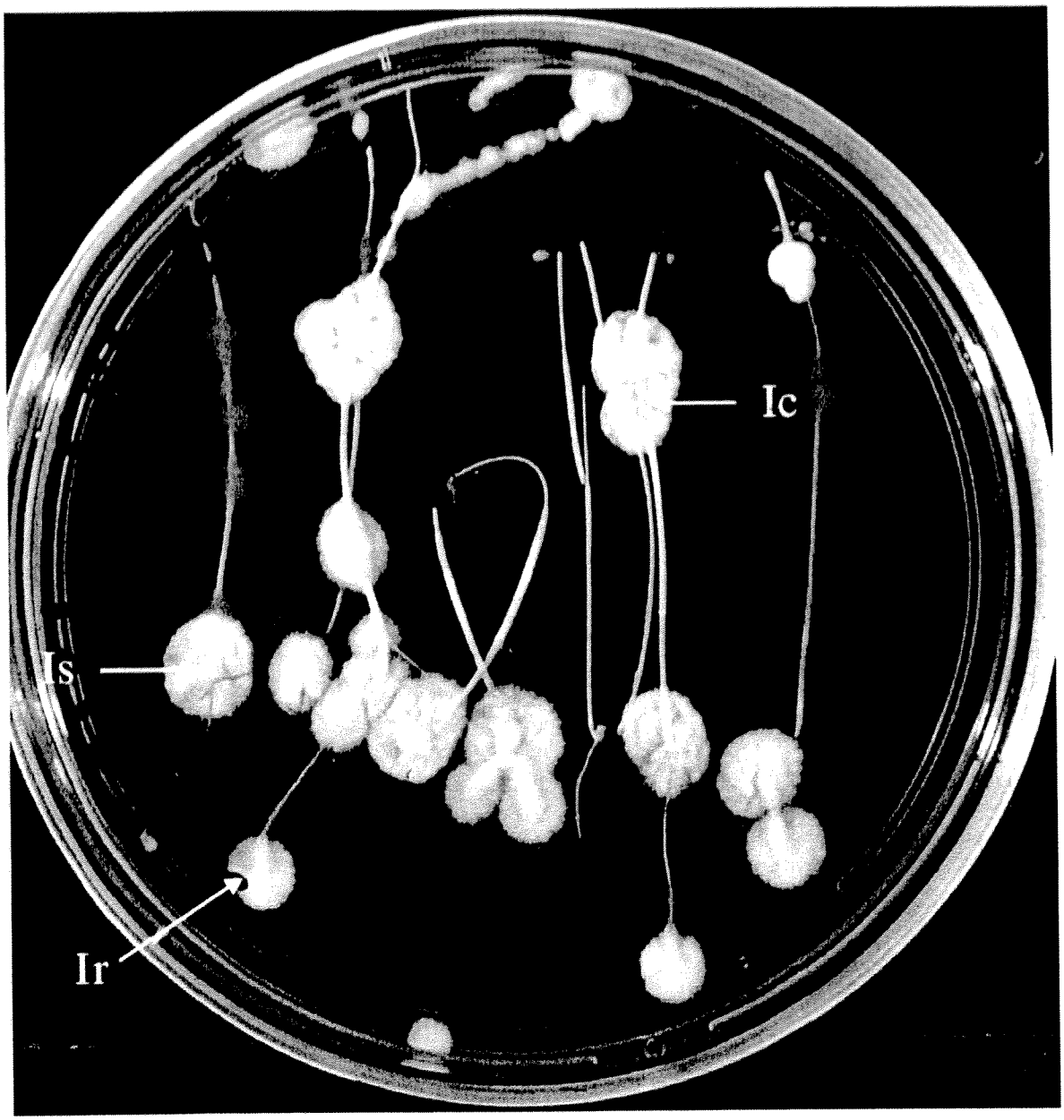

Fig. 1 Isolation of endophytes from germinating seeds of *Lolium perenne*. The fungus can be subcultured from the seed itself (Is), the coleoptile (Ic), the seminal roots (Ir).

L-asparagine: 2 g; KH_2PO_4: 1 g; $MgSO_4$, 7 H_2O: 0.5 g; KCl: 0.25 g; $FeCl_3$: 0.01 g; $CaCO_3$: 2 g; chloramphenicol: 0.05 g. The pH was adjusted to 6.0. The medium was distributed into Erlenmeyer flasks (100 ml in each) and sterilized in an autoclave (110°C for 30 min). Each isolate was represented by two replicates. The flasks were subjected to gentle shaking (70 rpm) in the dark. The temperature was 18°C for those isolates which, from their morphology, appeared to be *Gliocladium*-like, and 23°C for the other isolates.

The mycelium was removed after 3 wk and lyophilized. Extraction of the enzymes, gel preparation, electrophoresis and visualization were carried out according to the procedures described by Christensen *et al.* (1993), and Naffaa *et al.* (1998). The fungal species were identified from the description of electromorphs by the same authors.

Morphological description on PDA

Among the different morphological types which had been *a priori* subcultured from the isolations, only those that seemed stable in the course of the subsequent cultures were retained. The morphology of many isolates stabilized at the second

subculture even when variation was present in the first subculture.

These isolates were grown, observed and described in standard conditions: the culture medium was 20 ml of PDA distributed in Petri dishes 9 cm in diameter. Incubation was conducted in the dark, at 23 ± 0.5°C. The inoculum consisted of a mycelium disk cut with a 4-mm borer from the margin of a 1-month-old colony also grown on PDA. The Petri dishes were sealed with adhesive tape. The linear growth of the isolates was measured on the same Petri dishes. The diameter of each colony was measured after 45 d from the average of two perpendicular diameters. The growth rate was evaluated in mm d^{-1}. There were at least five replicates for each isolate.

Mycotoxin analysis

Lolitrem B was analysed by an adaptation of the HPLC method of Gallagher *et al.* (1985). Ground freeze-dried straw was mixed with chloroform-methanol (2 : 1) for 1 h on a horizontal shaker. After centrifugation, the supernatant was filtered and evaporated under a nitrogen stream. The dry residue dissolved in dichloromethane was cleaned on a Si Bond Elut column (Varian) conditioned with dichloromethane. Lolitrem B was then eluted from the column with dichloromethane-acetonitrile (80 : 20). The elution solution was evaporated under a nitrogen stream and the residue was dissolved in the HPLC mobile phase. The HPLC analysis was performed using a Thermoquest liquid chromatograph system equipped with a silica column (250 mm × 4.6 mm, 20 µl injection) and a fluorescence detector (excitation 268 nm, emission 440 nm). The mobile phase was dichloromethane-acetonitrile (85 : 15) with a flow rate of 1 ml min^{-1}. Calculations were performed with an external lolitrem B standard solution (supplied by R. G. Gallagher, AgResearch, New Zealand). The determined limit of quantification was 0.18 µg g^{-1} of dry plant material.

Ergovaline was analysed according to an adaptation of the method of Hill *et al.* (1993). Samples were extracted by alkaline chloroform with an internal standard (ergotamine 500 ng ml^{-1} in chloroform) and shaken for 2 h at room temperature. Extracts were filtered and purified on a silica column (Ergosil Analtech Newark, DE, USA) prewashed with chloroform. Pigments were removed by washing with chloroform-acetone (1 : 3). Ergot alkaloids were eluted with methanol and the eluent was evaporated under a nitrogen stream at 30°C. The dry residue was dissolved in methanol. The HPLC analysis was performed on a Thermoquest liquid chromatograph system equipped with a Si C-18 (Zorbax XDB) column (150 mm × 4.6 mm, 20 µl injection) and a fluorescence detector (excitation 250 nm, emission 420 nm). The mobile phase was acetonitrile (36, 5%) in aqueous ammonium carbonate (200 mg l^{-1}) run at 1.1 ml min^{-1}.

A standard was prepared by adding ergovaline (from F. Smith, Department of Pharmacal Sciences, Auburn University, AL, USA) and ergotamine (Sigma Chemical) to 500 mg of a noninfected ryegrass sample up to 500 ng g^{-1} and treated as described above. The limit of quantification of the method was 0.15 µg g^{-1} of dry plant material.

Peramine was analysed by a method supplied by B.A. Tapper (AgResearch, New Zealand). Dried ground straw was mixed with 30% propan-2-ol for 30 min at 90°C. After centrifugation, the crude extract was cleaned-up on a Bond Elut CBA column (Varian) conditioned with 80% aqueous methanol containing 2% ammonium hydroxide and then with pure methanol. Peramine was eluted from the column with a 5% formic acid – 80% methanol solution. The eluent was dried under a nitrogen stream and the residue resuspended in methanol. The HPLC analysis was performed using a Thermoquest liquid chromatograph system equipped with a silica column (250 mm × 4.6 mm, 20 µl injection) and an UV detector (280 nm). The mobile phase was a buffer consisting of 50 mM ammonium acetate, 5 mM guanidinium carbonate and 0, 2% acetic acid in 18% aqueous methanol at a flow rate of 1 ml min^{-1}. An endophyte-free ryegrass sample supplemented with peramine and extracted as described above was used as external standard for calculation. The limit of quantification of this method was 2 µg g^{-1} of dried plant material.

Statistical procedures

Linear growth data were subjected to variance analysis using the General Linear Model (GLM) procedure of SAS. In a first analysis, the main effect was the endophyte strains and the means were grouped by the cluster method developed by Scott & Knott (1974). The advantage of this method is to partitionate the mean values into nonoverlapping groups. In a second variance analysis, the morphological groups were designated as the main effect and the means were separated by the test of Bonferroni, which is adapted to unequal cell-size.

The production of lolitrem B for two distinct morphological groups of endophytes was compared by a two-sample *t*-test (procedure TTEST in SAS). The distribution obtained for the peramine was not Gaussian, leading to compare the medians by a nonparametrical test (proc. NPAR1WAY in SAS).

Results

Success of isolation

Isolates were obtained in all cases except one. The delay in the isolate emergence was extremely variable, from 7 to 33 d (median value, 14 d) but generally consistent within seed lots.

In 70 plants, the colonies growing from different seeds of the same lot were homogenous in their morphology and growth rate and were considered to belong to one isolate. However, for 12 plants, two different morphologies could be

distinguished among the colonies from the same seed lot. These differences in morphology remained stable when the colonies were subcultured. The two variants were considered as different isolates, distinguished by the letters A and B in Table 1. Thus, the total number of isolates studied was $70 + (12 \times 2) = 94$.

Species identification

Species identification was carried out by the isozyme patterns of MDH, PGI and PGM. All the isolates were identified by this method: 83 were found to belong to *Neotyphodium lolii*, 7 to LpTG-2 and 4 to *Gliocladium*-like (Table 1). Seeds from 12 plants harboured two different isolates. In one case, an isolate of *Gliocladium*-like was obtained as well as with an isolate of *N. lolii*. In three cases, isolates of LpTG-2 and *N. lolii* were obtained. In eight cases, two different isolates of *N. lolii* were found in the same seed lot.

Morphology of cultures on PDA

The isolates were assigned to eight stable and well-characterized Morphological Groups (MG) on PDA (Fig. 2).

Morphological Group I (MG I, Fig. 2a) consisted of slow-growing isolates which develop vertically more than horizontally. The mycelium is strongly aggregated. Vertical development is accompanied by the formation of irregular crests and convolutions. This morphology is often described as 'brain-like'. The margin of the colony is vertical. The colony is waxy, without aerial mycelium.

In MG II, the centre of the colony resembles MG I, but the margin is made of horizontal, smooth mycelium (Fig. 2b). Aerial mycelium is absent or scarce.

MG III is the most heterogeneous; it includes two subtypes which both are originally flat and smooth. In subtype A, domes or warts made of aerial, white mycelium erupt at the surface after 10–15 d of growth (Fig. 2c), they can later fill the centre of the colony or constitute sectors. In subtype B, the centre of the colony is white and slightly raised; after 15 d, it appears scored by furrows which are both radial and concentrical. These two morphologies have been included in one group because some isolates alternately showed one or the other morphology according to the subcultures.

MG IV is characterized by a high radial growth rate. The colony is flat and smooth, with very few or no aerial mycelium (some tufts in the central part). The margin, made of intramatricial mycelium, appears diffuse (Fig. 2d).

In MG V, as in the previous type, the colony is flat, with a diffuse margin. After 15 d, it is covered in its major part with aerial, powdery or granular mycelium (Fig. 2e). This group resembles group VIII (*Gliocladium*-like), however, the lower side is tinted with light grey, not with olive green.

The colony characterizing MG VI is flat, smooth, waxy, almost mucose, without aerial mycelium or white warts. The centre of the colony is typically grooved by curved, radial furrows (Fig. 2f).

The colony characterizing MG VII has an irregular outline. Its surface is cottony, and entirely covered with aerial mycelium. The margin is diffuse, consisting of superficial hyphae (Fig. 2g).

In the last group, MG VIII, the colony is circular, white, flat, and covered with powdery or granular aerial mycelium (Fig. 2h). The agar on the under side is tinted with olive green (permitting a clear distinction from group V).

The allocation of the 94 isolates obtained to Morphological Groups is shown in Table 1.

The seven isolates belonging to species LpTG-2 all belonged to MG VII and were the only isolates in this group. The four isolates identified as *Gliocladium*-like were the only ones in MG. The isolates identified as *Neotyphodium lolii* were allocated between MG I to VI, with the following distribution: MG I: five isolates; MG II: 26; MG III: 39; MG IV: five; MG V: four; MG VI: four. The six reference isolates obtained from AgResearch Grassland (R1–R6) had the following distribution: R1 and R2 (LpTG-2): MG VII; R3, R4, R5 and R6 (*N. lolii*): MG VI, V, V and I, respectively.

Growth rate

Analysis of the linear growth rate of the isolates led to the definition of nine nonoverlapping groups as shown in the last column of Table 1.

Figure 3 shows the distribution of linear growth for the eight Morphological Groups (MG). The main effect of MG on growth rate was highly significant ($P = 0.001$). According to the comparison of means, the different MG are classified in four groups of growth. MG IV and MG V belong to the group showing the significantly highest growth rate. LpTG-2 (MG VII) and *Gliocladium*-like (MG VIII) belong to a second group which overlaps the set including MG IV and MG V. MG II and MG III, which contain the majority of isolates, are classified in a third, distinct group of intermediate growth. MG I and MG VI constitute a fourth group characterized by significantly lower growth rates.

Mycotoxins

The analyses were carried out for a total of 79 plants representing 79 populations. For lolitrem B, the results of the 12 plants from which two different fungal isolates had been isolated were considered separately. Among the 67 plants which harboured only one isolate 11 did not contain lolitrem B and 56 contained lolitrem B at concentrations higher than the quantification threshold (0.18 $\mu g\, g^{-1}$). Of the 11 plants without lolitrem B, three plants harboured a *Gliocladium*-like fungus; that is no plant harbouring only a *Gliocladium*-like isolate synthetized lolitrem B. A further four plants harboured an isolate of LpTG-2. That is, no plant harbouring only an isolate

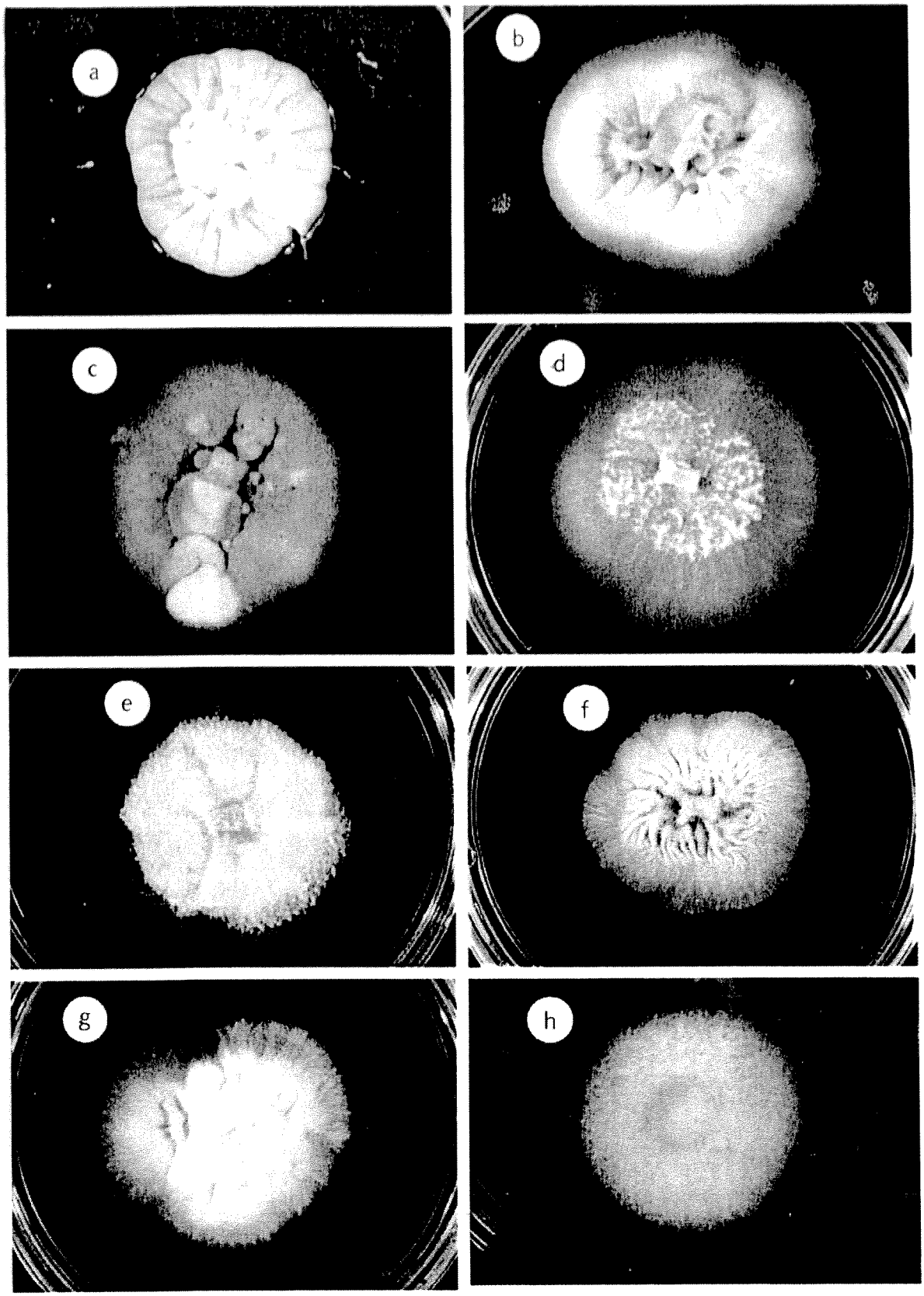

Fig. 2 Macromorphology of the eight Morphological Groups (MG) on PDA: a–f: *Neotyphodium lolii*, g: LpTG-2, h: *Gliocladium*-like.

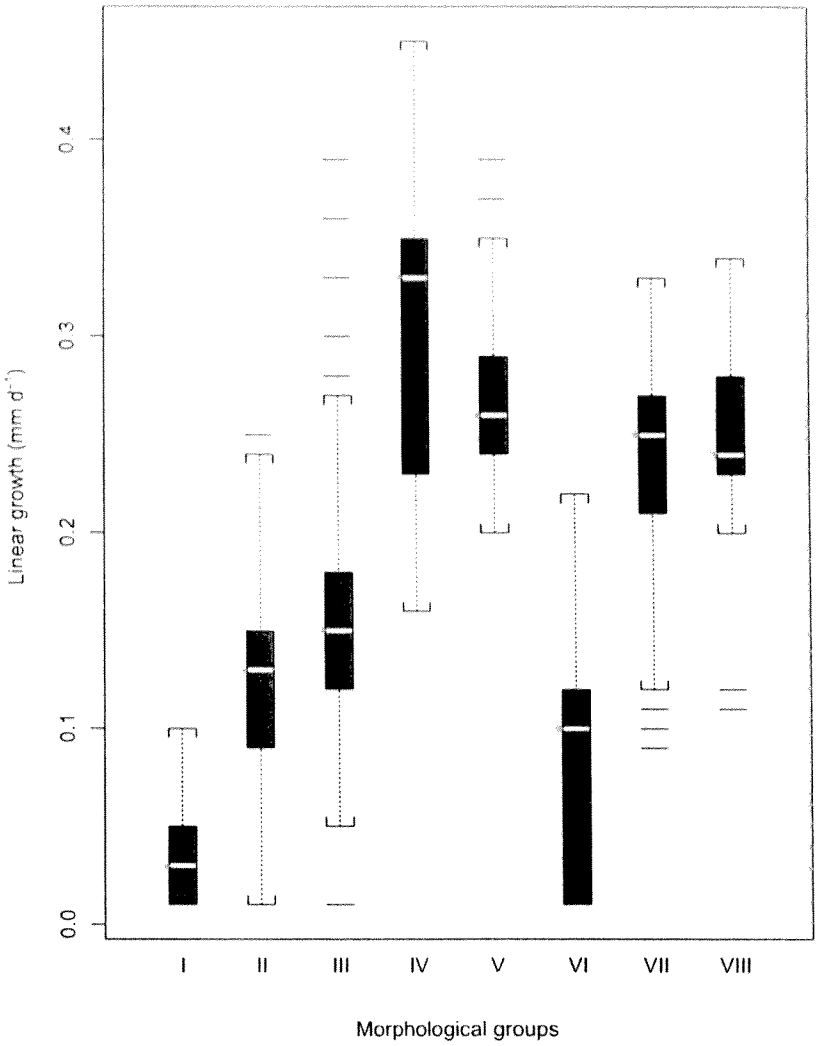

Figure 3 Relationship between increase in colony diameter (mm d^{-1}) and morphological groups. The horizontal, white line in each box is the median of the data; the height of the box is equal to the interquartile distance (IQD). The verticle line corresponds to a distance 1.5 × IQD from the median. Data points falling outside this interval are indicated by horizontal, black lines.

of LpTG-2 synthetized lolitrem B. Another four plants harboured an isolate of *Neotyphodium lolii* belonging to MG I. That is, no plant with an isolate in MG I synthetized lolitrem B.

The 56 plants containing lolitrem B did so at concentrations ranging from 0.8 to 5.75 μg g^{-1} (Fig. 4a) with an average value 2.90 μg g^{-1} All these isolates belonged to *N. lolii* and were distributed between MG II (22 isolates), MG III (31 isolates) and, marginally, MG IV (1) and MG V (2). MG IV and V were very small and the mean values for lolitrem B concentrations were compared only between MGs II and III, with MG II isolates producing significantly more lolitrem B ($P = 0.0063$).

In nine of the 12 plants from which two different isolates had been obtained, both isolates belonged to *N. lolii*. In these plants, lolitrem B was present at concentrations ranging from 0.80 to 4.30 μg g^{-1}. In three plants where there was one isolate of *N. lolii* and one isolate of LpTG-2 no lolitrem was detected in two cases, and one plant contained 2.1 μg g^{-1} lolitrem.

Among the 67 plants that harboured only one isolate, 41 contained ergovaline, at concentrations higher than the quantification threshold of 0.15 μg g^{-1} The plants without ergovaline included three plants harbouring *Gliocladium*-like isolates and 23 plants harbouring *N. lolii*. Among these 23 isolates, 22 belonged to MG II and only one to MG IV. Correspondingly, ergovaline was never detected in plants in which only one isolate of MG II had been found.

The 41 plants containing ergovaline, did so at concentrations ranging from 0.24 to 3.46 μg g^{-1} with an average value of 1.14 μg g^{-1} Among these, four belonged to LpTG-2 and 37 to *N. lolii*, and were distributed between MG I (four isolates), MG III (31 isolates) and MG V (two isolates). Interestingly, the highest concentrations were observed in two plants harbouring a MG I isolate.

For the plants containing two different isolates, in three cases a LpTG-2 isolate was associated with an isolate of *N. lolii* of MG III or MG IV, and these plants contained ergovaline. Four associations involved two isolates of *N. lolii* belonging to MG groups other than MG II and ergovaline was also present. In four situations in which MG II was involved in association

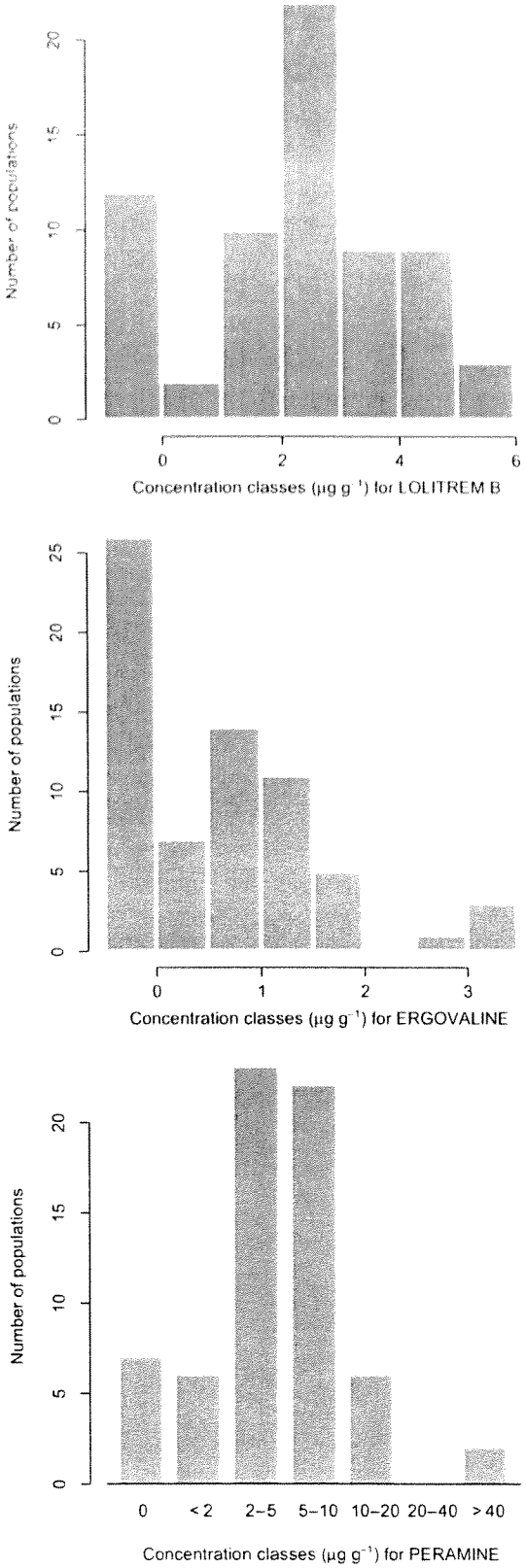

Fig. 4 Frequency distribution of mycotoxin concentrations. (a) lolitrem B; (b) ergovaline; (c) peramine.

with isolates of other groups, ergovaline was absent in three cases and present in one case. The last association was between *Gliocladium*-like and an isolate of *N. lolii* MG III and the plant contained ergovaline.

The quantification threshold was higher for peramine than for the other two mycotoxins (c. 2 µg g^{-1}). Thus, in some cases peramine was detected, but could not be quantified. Among the 66 plants which harboured only one isolate, seven had no detectable peramine, six showed only traces of the mycotoxin (concentrations < 2 µg g^{-1}) and 53 contained higher concentrations. The absence of peramine was not linked to absence or presence of ergovaline or lolitreme and could not be related to a special morphology of the isolate in culture. 53 plants contained peramine at concentrations ranging from 2.0 to 52.8 µg g^{-1}, according to an asymmetrical distribution, with a median value 6.30 µg g^{-1}. (Fig. 4c). Two plants showed a particularly high concentration of peramine (52.8 and 41.3 µg g^{-1}). Peramine production was significantly higher for the isolates of MG III than for those of MG II.

For those plants from which two different isolates had been obtained, the peramine concentrations showed no consistent pattern.

Discussion

Isolation of endophytes from germinating seeds was very successful. The 94 isolates obtained belonged to the three endophyte species which had already been described in *Lolium perenne*: *Neotyphodium lolii*, LpTG-2 and the '*Gliocladium*-like' fungus. *N. lolii* was the most common, representing 90% of the isolates. No other species were detected. The specific identification of these three species was based on their isozyme patterns for the two enzymes MDH and/or PGM, as described by Christensen *et al.* (1993) and Naffaa *et al.* (1998). The presence of conidia in culture for LpTG-2 at 20–24°C (*N. lolii* is always sterile at this temperature), is another criterion advocated to distinguish between these two clavicipitaceous species (Christensen *et al.*, 1993). The nine isolates of LpTG-2 isolated in our study were checked for the presence of conidia on PDA at 23°C. Conidia were observed for four isolates only. On the other hand, they were not observed on the cultures of five isolates of *N. lolii* which had been drawn by lot. The presence of conidia in cultures of LpTG-2 at room temperature seems to be a sufficient, but not necessary, condition for identification.

Pure culture macromorphology may be useful for species identification, since LpTG-2 and *Gliocladium*-like show a specific and very homogenous morphology (MG VII and MG VIII, respectively). *Neotyphodium lolii* varies greatly in morphology and in growth rate. Linear growth was preferred to biomass production as being potentially more discriminant. The strong correlation observed between macromorphology and linear growth is not surprising: the very slow apical growth of the mycelial hyphae of some strains is concomitant with a

high rate of branching, the result being a three-dimensional growth of the colony, which is stroma-like, being as high as wide. This morphology, which has sometimes been named 'brain-like', characterizes MGs I and II; in MG II, the central prosenchyma is surrounded by a band of flat mycelium while in MG I, it falls abruptly into the culture medium. These two groups are clearly distinct from each other and from all the other morphological types which can be described within *N. lolii*. The morphologies I and II appear stable with subculturing and are retained after a storage at low temperatures (−80°C). The flat aspect of the young colony characterized all the other morphological groups described; the MGs IV, V and VI show typical morphological aspects, and contain only a few isolates. MG III is less homogeneous; in this group, the centre of the colony can undergo different development after 20 d of growth: for example appearance of cottony warts or of radial and concentric furrows. This type is the one that contained the highest number of isolates. The typical appearance of the colonies of LpTG-2 on PDA has already been described by Christensen *et al.* (1991). These authors had also reported the high variability of the isolates of *N. lolii* and published photos resembling our groups II and III.

The variability within *N. lolii* was also considerable with respect to the synthesis of the three main mycotoxins: lolitrem B, ergovaline and peramine. Four isolates, all of French origin (6% of the isolates) did not synthetize lolitrem B. 23 isolates (37%) did not synthetize ergovaline. Of these 23, 20 originated from France, one from Poland, one from Yugoslavia and one from Greece. Six isolates (10%) (excluding those with traces of peramine lower than the quantification threshold) did not synthetize peramine. Among these six, all of French origin, one was also lolitrem B-deficient, two were ergovaline-deficient, and three synthetized both ergovaline and lolitrem B.

The most striking observation was the close link between the deficiency for ergovaline or lolitrem B in *N. lolii* and the morphology of the isolates: all four isolates which did not produce lolitrem B were from MG I and no isolate of MG I produced lolitrem B. Among the 23 isolates that did not produce ergovaline, 22 belonged to MG II. In MG II, no isolate produced ergovaline. By contrast, the peramine-deficient strains distributed among several morphological groups, including MG I and MG II.

As the MG I and MG II morphologies coincide with a slow linear growth rate, it seems that the 'mutation' (in the broad sense) which led to the absence of synthesis of lolitrem or ergovaline is regularly accompanied by disturbances of the mycelial growth. The loss of the ability to synthetize ergovaline (or lolitrem) could be the result of a chromosome deletion which could also result in the loss of genes playing a role in mycelial growth and morphogenesis. As the deficiencies for ergovaline and lolitrem are independent and accompanied by different morphological disturbances, the two deletions probably concern different chromosomes or chromosome fragments. The loss of a minor chromosome (a 'B chromosome', according to Kisler & Mia (1992)) could also be an explanation: Kuldau *et al.* (1999) found eight chromosomes in *N. lolii* of which three were smaller than 3 Mb.

Figure 4 shows the frequency distribution of the concentrations of the three mycotoxins in the plants. The concentrations of lolitrem B and ergovaline are similar to those in the literature (Di Menna *et al.*, 1992; Ball *et al.*, 1997b; Lane *et al.*, 1997a, 1997b). By contrast, the concentrations of peramine (median value: 6.3 $\mu g\, g^{-1}$ for 53 isolates) are lower than those in the literature, which are often in the range of 10–30 $\mu g\, g^{-1}$ (Ball *et al.*, 1995a, 1995b, 1997a). However, for two particular populations, the concentrations were much higher (> 40 $\mu g\, g^{-1}$). The genotype of the fungus, which controls the synthesis of a given mycotoxin, can also play a role in fixing the quantitative level of this mycotoxin within the plant. However, many other factors probably play a role, such as the host genotype, the mycelium density in the host tissues and various environmental factors; so it is difficult to assert that differences recorded in the three mycotoxins concentrations faithfully reflect genetic quantitative differences between the fungal isolates.

The isolates of MG II (which do not produce ergovaline) produced significantly more lolitrem B than the isolates of MG III. The isolates of MG III produce significantly more peramine than those of MG II. However, there was no correlation between the concentration of the three mycotoxins if all isolates are considered together.

Isolates deficient in both ergovaline and lolitrem B were not found. The predicted frequency of such strains would be the product of the individual frequencies: 6% × 37% = *c.* 2%. This low predicted frequency could explain why no such isolate was found. On the other hand, if it is true that both the ergovaline-free and the lolitrem B-free isolates of *N. lolii* are the result of genetic accidents (for instance of chromosome deletions), one would expect that the isolates carrying a double deletion would be particularly deficient and find it difficult to survive in the plant. However, screening of more than 1000 populations in New Zealand, has succeeded in finding such isolates (Fletcher & Easton, 1997).

The close link between the absence of ergovaline or lolitrem B and a specific morphology of the colonies in pure culture could, if it is confirmed on a higher number of strains from more various origins, considerably improve the process of selection of harmless strains. Such a selection could be carried out by laboratories equipped only for routine mycology and lacking the complex equipment and technical competence necessary for the analysis of mycotoxins by HPLC.

This study confirmed that the species LpTG-2, of hybrid origin (Schardl *et al.*, 1994), is in a minority among the endophytes of *L. perenne* in France. Only three French isolates of this taxon were found in our study: two from the region of Marseille and one from Corsica. Four other isolates were isolated from populations from Italy and Spain. The two reference isolates obtained from AgResearch also have a

Western Mediterranean origin. Thus, LpTG-2 appears to be a West-Mediterranean species.

The seven LpTG-2 isolates were homogeneous in their morphology, growth rate, isozyme pattern and mycotoxin synthesis (lolitrem B is not synthetized). The isozyme patterns MDH and PGM allow a certain identification of the species, but the morphology of the colonies on PDA may appear to be a safe method of identification.

The taxon '*Gliocladium*-like' was also a minority among our isolates. The four isolates obtained appeared identical as concerned their isozyme pattern, macromorphology (MG VIII type), high growth rate, low isolation delay, and preference for cool temperatures (18°C). The populations concerned came from Northern France, Central France and Germany. As for LpTG-2, the identification can be easily carried out from the macromorphology alone. Neither conidiophores nor conidia were observed *in planta* or in culture.

In 12 cases (14%) two different endophytes were isolated from the same seed lot. As each lot had been harvested from only one plant, two different fungal isolates must have coexisted within the same plant. In one case, a *Gliocladium*-like fungus was found associated with an isolate of *N. lolii*. In three cases, the association was between LpTG-2 and *N. lolii* and in eight cases, between two different isolates of *N. lolii*. Indeed, we could only detect the situations in which the two associated isolates of *N. lolii* belonged to two different MGs. It is probable that other situations exist in which the two (or more) isolates are morphologically similar and could be detected only with molecular markers.

Coexistence between two endophytes in the same plant has already been reported for the associations between a clavicipitaceous and a nonclavicipitaceous endophyte (Schmidt, 1994; Siegel *et al.*, 1995). The association between two different isolates of the same species of endophyte has already been reported and analysed by Meijer & Leuchtmann (1999) on the pair *Brachypodium sylvaticum*/*Epichloë sylvatica*. However, to our knowledge, the present work is the first mention of the presence of two different strains of *Neotyphodium lolii* in the same plant of *Lolium perenne*. Frequent coexistence between two clavicipitaceous endophytes in the same host may support parasexual hybridization in the continuum existing from parasitic *Epichloë* to mutualistic *Neotyphodium*, leading to new species.

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FUNGAL ENDOPHYTES OF GRASSES

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INTRODUCTION

The term endophyte (Greek: *endo* = *within* + *phyte* = *plant*) has been defined as an organism contained or growing (entirely) within the substrate plant, whether parasitically or not (100, 106). Using this term in its broadest sense, the subject of this chapter could include all fungi that spend all or nearly all of their life cycles in the host grasses. We do not discuss such endophytic fungi as the smuts and vesicular-arbuscular mycorrhizas that infect grass. Instead, our interests lie primarily with a specific group of clavicipitaceous fungi that belong or are related to fungi in the tribe Balansiae (5, 24). These fungi either are true endophytes that never produce external fructifications on the plant or else may produce external mycelium and/or spores that affect flower and seed production. We focus specifically on those grass–endophyte complexes that may also cause maladies of grazing animals.

Using the above criteria, two recent events involving grass–endophyte associations have important implications for the livestock industry. In 1977 Bacon et al (9) reported the close association of an endophyte *(Sphacelia typhina)* in infected tall fescue *(Festuca arundinacea)* and the incidence of

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fescue toxicosis (summer syndrome) in cattle. A similar association between a then unidentified endophyte in perennial ryegrass (*Lolium perenne*) and the incidence of ryegrass staggers in sheep in New Zealand was reported in 1981 by Fletcher & Harvey (27).

Since the initial work of Bacon et al (9) in 1977, researchers have come to further understand the relationship between fungal endophytes of grasses and animal toxicoses. This understanding includes the origin and incidence of infected grasses, modes of dissemination of the fungi, identification of the chemicals responsible for toxicoses, and control of the fungi. Specifically, what has been learned is that endophyte-infected tall fescue and perennial ryegrass are widely distributed in the United States and New Zealand, respectively; endophytic fungi are only seed disseminated; specific chemicals are responsible for fescue toxicosis and ryegrass staggers; infected pasture grasses cost the livestock producer hundreds of millions of US dollars annually in lost production; the fungi cannot be controlled in the field, but endophyte-free cultivars do result in greatly improved livestock production in the United States; and lastly, the relationship between grass and symbiont is primarily mutualistic, resulting in many benefits to host and fungus (5, 10, 97). With regard to this final point, it is now recognized that these grass-endophytes can play an important role in survival of the host plants subjected to environmental stresses (insects, grazing animals, drought, and heat). The potential for improved insect resistance of endophyte-infected grasses has already been exploited by the selection of naturally infected cultivars of *Festuca* and *Lolium* species (29, 30).

This chapter, therefore, reviews the harmful and beneficial effects of endophytic fungi on forage and turf grasses and on grazing animals. It includes a historical perspective on the fungi (incidence, dissemination, and control), the relationship between infected grasses and animal toxicoses, the nature of the grass–fungus interaction, and the value of infected and noninfected cultivars in pasture and turf.

HISTORICAL PERSPECTIVES

The presence of endophyte mycelium in the seed of a grass (*Lolium temulentum*) was first recorded by Vogl in 1898 (105), but the earliest known specimen of this host and fungus dates back 4,400 years to seeds found in the tomb of a Fifth Dynasty Egyptian Pharoah (64). Vogl's (105) discovery aroused interest because seeds of *L. temulentum* were known to be toxic to animals, and there was speculation that the fungus was responsible. Other species of *Lolium* were also found to contain mycelium in a high proportion of their seeds; thus, the endophytic nature of the fungus in plants was established (28, 76).

Classification

Endophytes of grasses and sedges are grouped in the tribe Balansiae of the Clavicipitaceae. Within this tribe are the genera *Balansiopsis*, *Atkinsonella*, *Myriogenospora*, *Balansia*, and *Epichloe* (24, 66). The taxonomy of endophytes, for which no teleomorphic state has been found, is still being debated. Morgan-Jones & Gams (70) created the section *Albo-lanosa* in the genus *Acremonium* to accommodate the endophyte commonly found in tall fescue. They named this fungus *A. coenophialum*, and subsequently, endophytes in other species of *Festuca* and *Lolium* have been placed in this section (59, 116, 117). These endophytes may be related to the clavicipitaceous teleomorph *Epichloe* (59, 70), but they have temporarily or permanently lost the ability to form sexual spores. Rykard et al (91) argue that it would be preferable to leave these newly named *Acremonium* species in the genus *Sphacelia*, the conidial state of *Epichloe typhina*. This question will only be resolved when the teleomorphic states of the fungi are found.

Latch et al (59) have described two seed-borne endophytes in perennial ryegrass and tall fescue with *Gliocladium*-like and *Phialophora*-like conidia, respectively. The relationship of these endophytes to those in the Balansiae is unclear.

All these Balansiae, except for *Myriogenospora*, produce systemic infections in leaves, culms, and inflorescences, but most become conspicuous only when sporulation occurs as the plant flowers. Many endophytes prevent their host from flowering, whereas others do not interfere with the plant's reproduction, and indeed, their mycelium may be present in the seed. Intercellular hyphae have been found in the ovary wall and throughout the nucellar tissue of *F. versuta*, tall fescue, and perennial ryegrass ovules (18, 65, 78). However, in some perennial ryegrass megagametophytes, hyphae of *Acremonium lolii* (59, 61) become intracellular when they breach the embryo sac and enter the antipodal cells (78). As the embryo enlarges and differentiates, the hyphae pass into the shoot apical region. Thus, some endophytes are very efficiently dispersed with their host seed and apparently have no need for spores as a means of dissemination. Indeed, it is believed that the only means of dissemination of *Acremonium* endophytes is through maternal transmission in infected seed (4, 54, 96).

Incidence

Species of *Balansia*, *Balansiopsis*, *Atkinsonella*, and *Myriogenospora* infect a wide range of grasses commonly found in the tropics that have a C_4-type photosynthetic pathway. Most of these grasses are regarded as weeds or as having minor agricultural importance (24, 66). *Epichloe typhina* is found on important genera of forage and turf grasses such as *Agrostis*, *Dactylis*, *Festuca*, *Holcus*, *Hordeum*, *Lolium*, and several other genera of minor agri-

cultural importance. The striking choke symptom of mycelium around the inflorescence is present on most infected plants (24, 92).

Endophytes that have *Acremonium*, *Sphacelia*, *Gliocladium*-like, or *Phialophora*-like anamorphic states and that produce no external fructifications on grasses were originally found only in species of *Festuca* and *Lolium* (9, 59, 70). Recent searches have extended the host range to include species of *Bromus*, *Poa*, and *Stipa* (111, 115, 117). Undoubtedly, further searches will extend this host range.

The incidence of endophytes in tall fescue, perennial ryegrass, and Italian ryegrass (*Lolium multiflorum*) has been studied because these grasses are of prime importance in many countries. Tall fescue is grown on 12–14 million hectares in the United States, and the two ryegrasses are the main pasture grasses sown in Europe and New Zealand. Endophytes have been found in seeds of these three grasses collected from areas of Europe where the species are indigenous and may possibly have originated (61, 96). The seed-borne nature of endophytes has resulted in their being spread, along with their hosts, throughout the world. Over 90% of tall fescue pastures in the United States contain plants infected with *Acremonium coenophialum* (97, 108). A survey by the Auburn University fescue diagnostic laboratory of samples from twenty-six states showed that 94% of pastures contained endophyte, and the plants in these pastures had a mean infection of 58% (95). The dominant cultivar of tall fescue in the United States is Kentucky-31, and most plants are infected with endophyte (96); however, new cultivars with a low incidence of endophyte have now been developed (97).

Seed samples from 53 of 64 old pastures of perennial ryegrass from eight European countries were found to be infected with the endophyte *A. lolii* (61). Recently sown pastures probably have a much lower incidence of endophyte because a survey of sixteen current European cultivars showed that only four contained endophyte, the average incidence being 15% in these cultivars (61). In New Zealand, the majority of plants in seven million hectares of perennial ryegrass are infected with *A. lolii*, and the two most commonly sown cultivars have 65–95% of the plants infected (54, 94, 97). There is little information on the incidence of an *Acremonium*-like endophyte in Italian ryegrass pastures. In Europe it appears to be low, especially among recent cultivars, but in New Zealand over 50% of plants of new Italian ryegrass cultivars may be infected with this endophyte (57, 61).

Methods of Detection

Grasses infected with endophytes that have as their anamorphic state *Acremonium*, *Gliocladium*-like, or *Phialophora*-like conidia are symptomless. The mycelium is intercellular within the host plants, but several techniques are available for detection and identification of these endophytes.

STAINING OF MYCELIUM Grasses infected with endophytes have hyphae in the leaf sheaths. If the adaxial epidermis is removed and stained, the septate, intercellular mycelium can be seen with the aid of a light microscope (16, 55). Hyphae of *Acremonium* spp. and *E. typhina* run parallel to the leaf-sheath axis and are seldom branched, whereas hyphae of the *Gliocladium*-like and *Phialophora*-like endophytes are branched profusely and present in greater quantities than those of the *Acremonium* spp. (59). Most of the endophytes can be found in all above-ground parts of the plant, and hyphae of the *Gliocladium*- and *Phialophora*-like spp. are also present in roots (59). Some endophytes prevent seed formation, but with others, viable seeds are produced that contain a dense mat of mycelium in the region of the aleurone layer (28, 69). This mycelium can be detected by softening seeds with sodium hydroxide or nitric acid, followed by degluming, squashing, and staining for microscopic examination (16). Unfortunately, the technique does not indicate whether mycelium is viable. In order to demonstrate endophyte viability, seeds must be germinated and the 3–4-week-old seedlings examined for mycelium (59, 108). A staining technique that distinguishes between living and dead mycelium in seeds would be of great value.

SEROLOGY Antisera have been prepared to cultures of *A. coenophialum* (48) and *A. lolii* (72) and used in an enzyme-linked immunosorbent assay (ELISA) to detect antigens of the fungi in tall fescue and perennial ryegrass. ELISA based on antibodies produced against mycelia of these two fungi does not differentiate serologically between *A. coenophialum*, *A. lolii*, *Acremonium*-like spp. in Italian ryegrass, and *E. typhina* (49, 74; D. R. Musgrave, personal communication). Musgrave et al (74) suggest that the major soluble antigen is a polysaccharide moiety of a protein lipopolysaccharide complex. They speculate that purified soluble antigens could be used to produce specific antisera for each of the endophytes. The ELISA technique is particularly useful for determining the location and quantity of mycelium in infected plants and for detecting endophyte mycelium in seeds (44, 72). However, difficulties experienced in using the technique have led to a decrease in its use for endophyte detection, and most endophyte diagnostic laboratories now use the staining technique (95, 108, 109). ELISA does not differentiate between viable and nonviable mycelium, so seed must be germinated and the young plants tested by ELISA or stained to verify the viability of mycelium in seed.

CULTURE Fungal endophytes of grasses can be cultured on artificial media, and most have been induced to sporulate. In some plant tissues, and especially in seeds, it is difficult to identify endophytes in situ, and so it is necessary to culture the fungi. Many endophytes grow very slowly in culture and are easily overrun by contaminating fungi and bacteria. Hence, culturing endophytes

from infected plant tissues is not used as a routine method of endophyte detection, but rather as an adjunct for endophyte identification. It is relatively easy to isolate and culture endophytes from leaf sheaths, nodes, and stem pith (9, 16, 55, 116), but isolating endophytes from seeds is more difficult, especially from seeds heavily contaminated with saprophytic fungi. The method most commonly used is to surface sterilize seeds in sulfuric acid (59, 108), but even this drastic treatment does not remove all fungi from heavily contaminated seeds. There is evidence (108) that this treatment kills the endophyte in some seeds. Thus, a more satisfactory surface-sterilization procedure is required.

INSECT BIOASSAY When given the choice of endophyte-infected and endophyte-free grass, some species of insects will feed only on the endophyte-free grass (31, 47, 58, 86). A relatively clear-cut preference for endophyte-free tall fescue and perennial ryegrass was shown by several species of aphids (47, 58). This finding led Latch et al (58) to advocate the use of *Rhopalosiphum padi* as a bioassay to detect tall fescue plants infected with *A. coenophialum* for large-scale, rapid screening of this endophyte in seedlings and mature plants.

Methods of Control

As previously mentioned, it is thought that endophytes of ryegrass and tall fescue are transmitted only through seed and that infection of endophyte-free grasses does not occur in nature (96, 119). Hence, if endophyte mycelium in seeds can be killed without harming the seed, or if mature infected plants can be freed from endophyte infection, then these plants will remain free of endophyte and will set endophyte-free seed. Several methods of killing endophytes in seeds and plants have been devised.

STORAGE OF SEED Viability of *A. lolii* and *A. coenophialum* mycelium in seed declines during storage (55, 75, 98). Neill (75) found that endophyte in perennial ryegrass seed was dead after two-years storage at ambient temperature, but it was shown subsequently that endophytes in perennial ryegrass seed remained viable for at least fifteen years if stored at low temperature (0–5°C) and low humidity (25–50%) (54). Recent studies have quantified the effect of environmental conditions and demonstrated that low temperature and low seed-moisture content will preserve endophyte viability (88, 108). If it is necessary to kill the endophyte but not greatly harm seed viability, the seed should be stored at high temperatures (30°C) and at a seed-moisture content of 8–10%.

HEAT Soaking seeds of perennial ryegrass and tall fescue in hot water for short periods of time killed the endophytes within them, but seed germination

was reduced by up to 50% (55, 98, 118). A complete kill of endophyte with only a minor reduction in seed viability was obtained when tall fescue seed was kept in hot moist air (49°C) for seven days (98).

CHEMICAL Endophyte mycelium in seeds of perennial ryegrass and tall fescue has been killed by applying fungicides that inhibit ergosterol biosynthesis, such as propiconazole, prochloraz, and triadimefon, to seeds (55, 98, 119). High application rates of fungicides are required for complete control of endophytes, and some stunting and death of seedlings are inevitable. Higher rates of fungicide application are required to kill mycelium of *A. coenophialum* in seed of tall fescue than those needed for *A. loli* in perennial ryegrass. These fungicides have been less effective in controlling endophytes growing in plants. Repeated foliar applications of triadimefon to plants grown in a greenhouse reduced the amount of *A. coenophialum* mycelium in tall fescue, but did not eradicate the fungus (98, 119). Granules of propiconazole applied to the soil in pastures of tall fescue suppressed the endophyte for many months, but did not eradicate it (119). Complete eradication of endophyte in perennial ryegrass and tall fescue has been accomplished by growing plants in sand and drenching benomyl around the roots (55; G. C. M. Latch, unpublished data), and in tall fescue by using bitertanol or propiconazole granules and foliar sprays (98, 119). With the fungicides available at present, it is clear that eradication of endophytes from infected pastures is not economically feasible. There is, however, a great need for a fungicide that could inexpensively accomplish that purpose.

Plants and seeds from which endophytes have been eliminated by any of the three methods just described will produce endophyte-free seed that can then be used as mother stock to build up endophyte-free lines. Thus, the simplest and most effective way of controlling animal disorders that result from grazing endophyte-infected grass is to establish pastures from endophyte-free seed. In the United States several new cultivars of tall fescue have recently been released and certified to have fewer than 5% of seeds infected with *A. coenophialum* (10, 44, 109).

ANIMAL TOXICITIES

As indicated in the introduction, renewed interest in endophyte-infected grasses began with a few key investigations into causes of animal maladies, specifically fescue toxicosis and ryegrass staggers (9, 27). Losses due to these disorders, in terms of animal production, have been estimated to be in the hundreds of millions of US dollars annually (82; J. A. Boling, personal communication). However, these estimates do not include losses in reproduction of cattle that may also be associated with these livestock disorders (36). Reproductive capacity of both female and male mammals appears to be

decreased on diets high in endophyte-infected forage or seed (77, 104, 121, 123).

Fescue Toxicosis

The poor cattle performance associated with *A. coenophialum*-infected tall fescue is characterized by reduced weight gain, decreased milk production, lower feed intake, rough haircoat, excessive salivation, increased respiration rate, and high rectal temperatures (40). These symptoms most often occur in, but are not limited to, the summer months. An additional malady of cattle associated with endophyte-infected tall fescue in some localities is characterized by lameness, ischemic necrosis, dry gangrene of the extremities, and, in severe cases, loss of hooves (35, 40). This latter malady is known as fescue foot and occurs most frequently during the winter, with the onset of clinical symptoms often linked to a sudden drop in ambient temperature.

Several alkaloids have been suggested to contribute to fescue toxicosis. The loline alkaloids, *N*-acetyl and *N*-formyl, have been shown to reach concentrations as high as 0.8% per unit dry weight in stems, leaves, and seeds of endophyte-infected tall fescue and have not been detected in endophyte-free tall fescue plants (15, 50). It has not been established if the loline alkaloids play a role in fescue toxicosis. However, these alkaloids do appear to serve as insect-feeding deterrents (45, 47).

The ergot alkaloids, clavine and ergopeptide types, are produced in vitro by *A. coenophialum* (5, 67, 80) and have been found in low concentrations (relative to the loline alkaloids) in leaf blades and sheaths of endophyte-infected tall fescue collected from pastures (67, 120). Attention has been primarily focused on ergovaline, an ergopeptide alkaloid that constitutes 10–50% of the total ergot alkaloid concentration in the forage (5, 67). Many of the symptoms of fescue toxicosis are consistent with signs of ergot poisoning (5, 68, 120).

Ryegrass Staggers

Perennial ryegrass staggers is a neurological disorder of animals that most commonly affects sheep, but also occurs in cattle, horses, and deer (71). Animals with this disorder appear normal until disturbed, at which time they suffer severe muscular spasms, usually resulting in collapse followed by an apparently quick recovery (71, 97). In severe outbreaks, deaths from falls or drowning are common (71). Ryegrass staggers occurs during the summer and autumn and is commonly associated with warm ambient temperatures and with grazing of short-grass swards (71). In addition to the staggers condition, animals grazing *A. lolii*-infected perennial ryegrass show reduced weight gains, which are probably attributable to reduced intake of herbage (26).

The prime suspect causative agents of ryegrass staggers are the lolitrem

neurotoxins (32–34). The major lolitrem neurotoxin, lolitrem B, is a lipophilic complex substituted-indole compound with a molecular weight of 685 and formula $C_{42}H_{55}NO_7$ (84). The lolitrems have not yet been reported to be produced by *A. lolii* in culture, but related tremorgenic toxins, penitrems, janthitrems, and aflatrem, are of fungal origin (34).

Toxicities Attributed to Balansia*-Infected Weed Grasses*

Many warm-season perennial weed grasses infected with species of *Balansia* may possibly be involved with some outbreaks of animal toxicosis (5, 6). Weed grasses parasitized by *Balansia* species include smutgrass (*Sporobolus poiretii*), broomsedge (*Andropogon virginicus*), lovegrass (*Eragrostis hirsuta*), and panicum (*Panicum anceps*) (5, 6). Bacon et al (5) reported that in the summer months a large percentage of a pasture may consist of a weed grass and that the majority of the weed grass may be infected by *Balansia* spp. Several species of *Balansia* have been shown to produce ergot alkaloids in vitro as well as in vivo (3, 5–8). Furthermore, cultures of *Balansia epichloe* isolated from infected smutgrass depressed serum prolactin concentrations in lactating Holstein cows following oral dosing for three consecutive days (107). Prolactin is a pituitary hormone that is necessary for the secretion of milk and that probably also plays a role in affecting the reproductive capacity of both female and male mammals (107). Depressed serum prolactin levels also occur in animals that have consumed *A. coenophialum*-infected tall fescue forage (12, 40, 42) as well as in sheep that have grazed *A. lolii*-infected perennial ryegrass (26).

GRASS–FUNGUS INTERACTIONS

Parasitic and Mutualistic Symbiosis

The endophytic fungi infecting grasses are biotrophic; that is, they obtain all their basic nutritional needs from living tissue. Furthermore, Lewis (62) considers fungi such as *E. typhina* and those responsible for "symptomless parasitism" as belonging to a group of ecologically obligately symbiotic biotrophs (obligate biotrophs). Lewis defined this group of fungi as "parasitic and mutualistic symbionts with no capacity for free living existence other than as gametes, cysts or as laboratory cultures." The terminology and classification are based on de Bary's (22) original concept of symbiosis: "Those which . . . feed on living organisms, whether plants or animals, are termed parasites. Their relationship with their hosts is that of a common life, a *symbiosis*." de Bary recognized that gradation of symbiosis existed and that parasites could either quickly destroy their host or that parasite and host could mutually and permanently live together and support one another.

According to Lewis's scheme, *E. typhina* and *Balansia* spp. would be

considered biotrophic and parasitic endophytes, whereas the *Acremonium* endophytes would be biotrophic and mutualistic symbionts. This scheme of classification of *E. typhina* is not absolute because isolates in some host grasses show gradations of symbiosis. Some isolates act as parasitic symbionts (24, 110), whereas others behave, e.g. the symptomless *Acremonium* endophytes, as mutualistic symbionts (29, 92). Whether a specific symbiotic association is parasitic or mutualistic can only be determined by comparing the fitness of the host and fungus when living independently with their fitness when living in association (63).

The major features of mutualistic symbioses are a lack of destruction of host cells or tissue; nutrient or chemical cycling between host and fungus; enhanced longevity and photosynthetic capacity of cells and tissue under the influence of infection; enhanced survival of the fungus; and a tendency toward greater host specificity than seen in necrotrophic infections (62). Researchers (5, 10, 82, 96, 97) have used this concept of mutualism to suggest that the *Acremonium* endophytes infecting perennial ryegrass and tall fescue enter into a relationship with their respective grasses that benefits both host and fungus. These benefits include enhanced dissemination and survival of the fungus, improved growth for the host plant, and tolerance to herbivore feeding (grazing animals and insects).

Benefits to the Fungus

Detection of endophytes by electron microscopy (25, 41), staining (9, 23, 108), and ELISA (72, 96) clearly indicates that intercellular fungal growth of *Acremonium* spp. and isolates of *E. typhina* is distributed unequally in the plant, with highest concentrations found in the leaf sheaths, seeds, and crowns. These regions can act as "sinks" for the accumulation of soluble nitrogenous and carbohydrate substrates (5). These substrates would have to leak out of the plant cells into the intercellular spaces and fluids for uptake by the endophytes. Whether the fungi induce leakage or possess translocational mechanisms for movement of plant products is unknown. However, reciprocal translocation of carbohydrates between host and pathogen has been reported in Bahia grass infected with *Myriogenospora atramentosa* (99). The nutritional requirements of the fungi in the plant are unknown.

In vitro rates of growth, pH and temperature optima, utilization of carbon and nitrogen sources, and synthesis of specific fungal products are known for some endophytes (3, 9, 20, 53, 59, 114). This information cannot be extrapolated to the in vivo situation, but can be used to compare nutritional, physiological, biochemical, and genetic potential of isolates (10). Clearly, the endophytes benefit from the association with their hosts by nutrition, long-term protection, improved dissemination (via seed), and survival.

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Benefits to the Host

GROWTH Perhaps of all the potential benefits to infected grasses, changes in plant physiology and patterns of growth are the most complex and difficult to assess. In studies in Kentucky over a four-year period, Siegel et al (96) did not find differences in forage (dry matter), seed yield, and stand persistence of Kenhy tall fescue in plots that ranged from 7–75% of plants infected with *A. coenophialum*. On the other hand, there are reports suggesting that endophyte-free cultivars do not survive as well as infected ones. In Texas, infected tall fescue that had been grazed produced more forage than plants in noninfected grazed pastures (87). In addition, during the third year of the trial many plants died in two of the three noninfected replicated plots. Funk et al (29) reported that over a seven-year period in New Jersey, endophyte-infected tall fescue turf plants outperformed noninfected turf plants from the standpoint of resistance to crabgrass invasion and recovery from summer drought stress.

The differences in the three reports are difficult to reconcile, but as will be shown, infected perennial ryegrass and tall fescue exhibit striking resistance to herbivores (insects and grazing animals). A combination of environmental stresses (heat and drought), herbivore feeding, and competition with other grass and weed species could account for the differences reported in growth and survival between infected and noninfected grasses. Biotic and abiotic stresses have been reported to be the reason for the inability of endophyte-free perennial ryegrass cultivars to survive in New Zealand (11, 82).

There are no differences in forage quality (crude protein, neutral detergent fiber, acid detergent fiber, and digestibility) between endophyte-infected and noninfected tall fescue (14). When infected tall fescue was grown under controlled conditions, the plants had greater rates of photosynthesis and a greater increase in fresh weight; they produced more tillers during regrowth; and they had a lower percentage leaf roll (during a drying cycle) than did endophyte-free tall fescue. Infected plants also used water more efficiently than did noninfected plants (L. P. Bush, personal communication). These differences would support the hypothesis that infected tall fescue plants have advantages over noninfected plants when grown during periods of environmental stress.

The clearest example of enhanced growth has been reported for *A. lolii*–infected perennial ryegrass grown under controlled conditions in New Zealand (60). Infected plants (when compared to noninfected) had significant increases in total leaf area, tiller numbers, and growth of leaves, but the shoot-root ratio was unaffected.

The chemical basis for the changes in physiology and growth patterns of *Acremonium*-infected plants is unknown. It is possible that members of the

Table 1 Species of insects reported to be affected by endophyte-infected *Festuca* and *Lolium* species of grasses[a]

Insect	Grass	Fungus	Reference
Crambus spp (Sod webworms)	PRG[b]	A.l.	31
Listronotus bonariensis (Argentine Stem Weevil)	PRG, TF[b]	A.l., A.c.	86, 101
Sphenophorus parvulus (Bluegrass billbug)	PRG[b]	A.l.	2
Spodoptera frugiperda (Fall armyworm)	PRG, TF	A.l., A.c.	38, 39
Acheta domesticus (House cricket)	PRG, TF	A.l., A.c.	1
Rhopalosiphum padi (Oat-birdcherry aphid)	TF	A.c.	47, 58
Schizaphis graminum (Greenbug aphid)	PRG, TF, HF[b], CF[b]	A.l., A.c., E.t., E.t.	47
Oncopeltus fasciatus (Milkweed bug)	TF	A.c.	47
Blissus leucopterus hirtus (Chinch bug)	CF[b]	E.t.	29
Heteronychus arator (Black beetle)	PRG[b]	A.l.	82
Draculacephala antica (Sharpshooter leafhopper)	TF[b]	A.c.	52
Chaetocnema pulicaria (Corn flea beetle)	TF[b]	A.c.	52

[a] Abbreviations: TF, tall fescue (*F. arundinacea*); PRG, perennial ryegrass (*L. perenne*); CF, Chewings fescue (*F. rubra* subsp. *commutata*); HF, hard fescue (*F. longifolia*); A.c., *A. coenophialum*; A.l., *A. loliae*; E.t., *E. typhina*.
[b] Based on field observations.
[c] M. Siegel, unpublished data.

Clavicipitaceae produce, or induce the plant to produce, auxinlike plant-growth regulators and/or plant growth inhibitors, or they may alter hormone metabolism. These are distinct possibilities when one considers that symptoms of infection by *E. typhina*, and other members of the Balansiae, include not only the inhibition of flowers and seed, but also enhanced plant growth and tillering, dwarfism, or deformation of the flag leaf (5, 17, 24, 37, 60, 110). Auxinlike indole compounds have been isolated in vitro from cultures of *Balansia epichloe* (79). The production of these compounds may reflect an alteration of nitrogen metabolism and assimilation in the infected plant (5).

TOLERANCE TO INSECT HERBIVORES Perhaps the most striking difference between *Acremonium*-infected and noninfected *Festuca* and *Lolium* species of grasses is the resistance by infected grasses to attack by insects (Table 1). It is

now known that *Acremonium*-infected grasses produce an array of chemicals that have a wide range of biological activity (5, 10, 14, 82, 97).

The compounds responsible for insect (Argentine stem weevil, *Listronotus bonariensis*) resistance in *A. lolii*–infected perennial ryegrass have been identified as a peramine alkaloid (89) and the lolitrem neurotoxins (84). Peramine (89, 90), or a peraminelike compound (85), is made in culture by *A. lolii*.

The ergopeptide alkaloids (predominately ergovaline) isolated from *A. coenophialum*–infected tall fescue (67, 120) have not been reported active against insects. However, clavine ergot alkaloids (ergotamine and ergonovine) do have insecticidal properties (D. L. Dahlman, personal communication). The pyrrolizidine alkaloids (*N*-formyl and *N*-acetyl loline) produced in *A. coenophialum*–infected tall fescue (13, 15, 43, 50, 51) have also been reported responsible for insect resistance (45, 47). These alkaloids have not been recovered from either mycelium grown in culture or viable fungal mats isolated from enzyme-digested seed (L. P. Bush, personal communication). A steroid tetraenone metabolite (ergosta-4,6,8-(14), 22-tetraen-3-one), biologically active in brine shrimp and chick embryo bioassays, has been isolated from infected tall fescue and cultures of *A. coenophialum* (9, 21). This compound has not been tested for activity against insects.

Techniques now exist for producing synthetic grass–endophyte complexes. This synthesis involves the transfer of different species of fungi to host grasses and grasses not known to be hosts by inoculation of one-week-old seedlings (via wounding of the meristem) (56), by inoculation of callus tissue (46), and by plant breeding (maternal-line selection) (29, 30). These techniques should assist in further characterization of the grass–endophyte relationship and biological activity of synthesized chemicals.

One may assume that if biologically active chemicals are of fungal origin, they could be produced in any naturally occurring as well as in synthetic grass–endophyte combinations. Conversely, if compounds are only produced in conjunction with certain grass–fungus interactions, then a greater degree of specificity of synthesis would be expected. Peramine is not only produced in *A. lolii*–infected perennial ryegrass, but in *A. coenophialum* and *E. typhina* naturally infected grasses as well (M. R. Siegel, unpublished data). In addition, peramine was found in two synthetic grass–endophyte combinations: an *A. lolii*–infected tall fescue and *A. coenophialum*–infected perennial ryegrass. There appears to be a much greater specificity of synthesis of the loline alkaloids in infected grasses. Synthesis occurred only in *A. coenophialum*–infected tall fescue and in the synthetic *A. coenophialum*–infected perennial ryegrass. These alkaloids could not be recovered from tall fescue artificially infected with *E. typhina*, *A. lolii*, or *Phialophora*-like endophytes. Loline alkaloids have been reported in *L. temulentum* (19);

however, it is not known whether the plant material was infected with the *Acremonium*-like species that has been isolated from this grass (57). These data still do not explain whether the host, fungus, or both are responsible for synthesis of the loline alkaloids. It has yet to be determined whether the ergot alkaloids and lolitrems can be produced by other fungi in their natural hosts or by the fungi in nonhost grasses.

The ability of endophyte-infected plants to produce biologically active compounds under field conditions may depend on the location and concentration of the endophytes in the plant. Synthesis of these compounds may depend on factors that affect plant and fungal growth. These include internal and external nutritional conditions and the external environment (light, water, and temperature). The fungus-produced toxins peramine (90), ergot alkaloids (5, 67, 81), and lolitrems (33, 34) (classified here as being of fungal origin because of their similarity to other mycotoxins) are found in relatively low concentrations (0.3–22 μg/g plant dry weight) in infected plants. On the other hand, the loline alkaloids, presumed to be of plant origin, are found in concentrations as high as 8,253 μg/g plant dry weight (50).

The distribution and concentration of the chemicals within the plant vary with the compound, the location of the endophyte in the plant, and the season. There is little information on peramine, other than that concentrations of 1 μg/g plant fresh weight were recovered from *A. lolii*–infected plants (89). Leaf sheaths and blades of *A. lolii*–infected perennial ryegrass contained lolitrems at concentrations as high as 4.9 and 0.37 μg/g plant dry weight, respectively (33). The amount of *A. lolii* mycelium in infected plants has been positively correlated with resistance to Argentine stem weevil and with the time of year when ryegrass staggers occurs (11, 27, 82, 83).

Total ergot alkaloid concentrations in leaf sheaths and blades of *A. coenophialum*–infected tall fescue plants were 22 and 3.5 μg/g plant dry weight, respectively (5, 67). Total ergot alkaloids increased 2.5-fold in infected tall fescue following application of nitrogen fertilizers (67). Furthermore, added nitrogen was associated with increased growth of endophyte in the plant and increased symptoms of animal toxicoses (67). High levels of loline alkaloids have been found in the same parts of the plant where high densities of the fungus are known to exist (13, 43, 50, 51). In greenhouse studies, the fungus concentration in the plant did not increase during initial growth, but increased in first and second regrowth tissues (13). Loline alkaloid concentrations followed the same pattern, except after the second harvest, when concentrations in the blade (where there was little fungus) nearly equalled those in the stem. This finding suggests that these alkaloids, or the signal for their synthesis, are translocated to the leaf blades, and hence, regrowth tissue in the field may contain large amounts of alkaloids. In greenhouse experiments, loline alkaloids increased 2–3-fold in plants sub-

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jected to moderate-to-severe water stress (51). However, in contrast to the ergot alkaloids, increased application of nitrogen did not increase levels of loline alkaloids (51). Concentrations of loline alkaloids were monitored in tall fescue during a three-year steer grazing study (103). They generally rose to their highest concentrations in August, and then remained constant until late fall. However, concentrations varied greatly from year to year, suggesting an effect of environment on alkaloid concentration in the plant.

There is little information on the effect of concentration of chemicals in infected plants and insect toxicity. Using the large Milkweed bug (*Oncopeltus fasciatus*) bioassay (47), D. J. Dahlman (personal communication) has found LD_{50} concentrations of ca 8 μg/ml for *N*-formyl loline, ergotamine, and ergonovine. Agar-plate bioassays of peramine with Argentine stem weevil indicated that feeding deterrence occurred at 10 μg/ml (89). No feeding deterrence occurred with lolitrems, but after 24-days feeding on 5 μg/ml, larval weights were reduced and 40% mortality occurred (84).

TOLERANCE TO GRAZING HERBIVORES In the section on animal toxicoses, we discussed the relationship between endophyte-infected grasses and animal maladies. One of the direct effects on animals grazing endophyte-infected grasses is a reduction in average daily weight gains. Of equal importance is the observation that infected tall fescue is tolerant to overgrazing by livestock (J. A. Boling, personal communication). The lack of overgrazing of the infected cultivars may be due to better growth of the plants and differences in agronomic factors between the infected and noninfected cultivars. More likely, it is due to the fact that animals feeding on infected perennial ryegrass and tall fescue consume substantially less forage than those feeding on noninfected grass (26, 102).

DISEASE RESISTANCE The last of the benefits of endophyte-infected grasses is the potential for disease resistance. White & Cole (113, 114) and M. R. Siegel & G. C. M. Latch (unpublished data) have demonstrated that isolates of *A. lolii*, *A. coenophialum*, unidentified *Acremonium* sp., *Phialophora*-like sp., and *E. typhina* from various grass spp. produced antibiosis against a range of fungal plant pathogens and saprophytic fungi in culture. The amount of antifungal activity and the spectrum of activity were found to be dependent on the isolate. Resistance to diseases in pasture or turf *Festuca* and *Lolium* spp. has not been reported.

Speculations on the Origins of Grass Endophytes

The recent origins of *A. coenophialum* and *A. lolii*-infected pasture and turf grasses have been discussed elsewhere (10, 96, 97). It is clear that Europe is the origin of these infected cultivated grasses. However, the determination

that endophytes not only exist in native *Festuca* and *Lolium* species (112, 114, 116), but in large numbers of species in the Poaceae (111, 115, 117) as well, raises important questions concerning the recent origins of these endophytes and also the evolutionary relationships between grasses and endophytes. Because of the wide geographic distribution of cultivated and native grasses infected with endophytes, White (111) has suggested that the vegetative phases of these fungi are, or have evolved from, species of the ascomycetous genus *Epichloe*. Mycological and serological data (49, 73) and evolutionary concepts of biotrophy (63) give some support to this hypothesis.

Lewis (63) considers the biotrophic habit of great antiquity and believes that the basic features of the evolution of parasitism and mutualism within biotrophy occurred at least 400 million years ago. The evolution from parasitism to mutualism therefore depends on the capacity of the partners to avoid or tolerate environmental stresses, as well as the obvious capacity for two-way cycling of nutrients and other prerequisites of biotrophy previously discussed. As was pointed out earlier, *E. typhina* appears to be in the process of evolution, as indicated by the various degrees of symbiosis that exist between different isolates and their hosts. The end result of this evolutionary process would then be fungi like the *Acremonium* spp., which act as mutualistic symbionts.

USE OF ENDOPHYTE-INFECTED AND NONINFECTED GRASSES

Once specific aspects of the nature of the host-fungus interaction were recognized, then the advantages of both infected and noninfected grasses were realized. The fact that the grass-endophyte relationship could be manipulated to remove or introduce fungi into different cultivars of grasses only served to accelerate their use. However, it is now apparent from a better understanding of the grass-fungus relationship that whereas some uses of endophyte-free cultivars are desirable, they may not be practical. Instead, further manipulations of infected grasses may be necessary to realize the full potential of improved productivity from animals grazing infested pastures.

Use of Endophyte-Free Cultivars

As soon as it was recognized that endophyte-infected grasses were responsible for animal toxicoses, it was suggested that removal of the fungi would lead to an alleviation of symptoms and improved animal productivity (9, 27). Eradication of *A. lolii* and *A. coenophialum* from perennial ryegrass and tall fescue cultivars, respectively, was accomplished by killing the fungi in plants or seed by storage, heat, or chemical treatments.

Some of the endophyte-free tall fescue cultivars were clearly superior to

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infected cultivars from the standpoint of improved animal production (93, 102). However, the endophyte-free perennial ryegrass cultivars used in pastures in New Zealand were susceptible to insect predation and drought, and often did not survive (11, 82). Whether the same phenomenon will occur with the newly released endophyte-free tall fescue cultivars is unknown and is a topic of considerable speculation.

What is unclear is whether the superior character of tall fescue is due to the endophyte grass complex or, in part, to the normal genetic complement of the plant. Consequently, it may be necessary to sow large areas of endophyte-free pastures before an appropriate evaluation of survival can be made.

Use of Endophyte-Infected Grasses

The confirmation of insect resistance, enhanced growth, and persistence of endophyte-infected grasses has led grass breeders to develop, by maternal line selection, naturally infected cultivars for turf (29–31). Seed of infected grasses used in turf will require special storage facilities and labeling to guarantee the viability of the endophytes.

Another approach to the use of endophyte-infected grasses would be to introduce specific fungal isolates, via wounding of seedlings, into host or nonhost grasses (synthetic grass–endophyte combinations). Assuming that the compounds toxic to animals are different from those that affect insects, it should be possible to develop synthetic combinations of host and endophyte suitable for turf and pasture. Insect deterrent compounds would be present in both turf and pasture cultivars. Turf grasses could still contain compounds toxic to animals, but pasture grasses would have little or none (10, 83, 89). Endophytes in these synthetic combinations could be naturally occurring biotypes, if suitable ones were available, or they could be genetically modified. All endophytes must, of course, be seed transmitted.

We have made a number of positive, but unproven, assumptions to support the use of modified endophyte-infected pasture grasses. We already know that the grass-endophyte system in some cases may contain more than one chemical responsible for insect resistance. This fact raises the question of what happens to insect resistance when one of several toxic compounds is not present in the modified synthetic combinations. It is obvious that it will be necessary to identify all the biologically active compounds and their spectrum of activity in plants, insects, and animals before new synthetic grass–endophyte cultivars are released for pasture use.

CONCLUDING REMARKS

Through interdisciplinary research, plant pathologists, chemists, agronomists, entomologists, and animal scientists have come to better understand a

unique grass–fungal endophyte relationship. It is now clear that in certain grass–fungus complexes, mutualistic relationships result in benefits to both partners. Understanding this relationship has resulted in the development and release of both infected and noninfected cultivars for use in pasture and turf.

In order to ensure the proper use of these newly developed cultivars, interdisciplinary research must continue into the nature of the grass–endophyte relationship. Questions of interest and importance that need to be answered include the mechanisms of synthesis and the spectrum of activity of biologically active compounds; the interaction of external and internal stimuli on synthesis of these chemicals and on growth of the endophytes and plants; biochemical mechanisms of pest resistance and animal toxicoses; and the nature of biotrophy and how it relates to the ecological fitness and survival of the endophytes and grass hosts.

The widespread distribution of fungal endophytes in grasses and the potentially mutualistic nature of the relationships may suggest an importance that approaches other plant–microorganism complexes such as those that involve the mycorrhizal fungi and nitrogen-fixing bacteria. The improvement of grasses used for pasture, turf, and conservation are long-term goals of plant breeders; a fuller understanding of grass–endophyte relationships will aid in fulfilling these goals.

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FIVE ENDOPHYTES OF LOLIUM AND FESTUCA IN NEW ZEALAND

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ABSTRACT

Five endophytic fungi were isolated from pasture grasses in New Zealand. A Gliocladium-like species and Acremonium loliae Latch, Christensen & Samuels sp. nov. were isolated from Lolium perenne. A Phialophora-like species and A. coenophialum were isolated from Festuca arundinacea. Epichloe typhina (Persoon : Fr.) Tulasne was isolated from leaves and seed of apparently uninfected plants of F. rubra and from immature stromata on seed heads of F. rubra. These endophytes are described and illustrated and their taxonomic relationships to previously reported but unidentified fungal endophytes of Lolium and Festuca are discussed.

INTRODUCTION

Details of the morphology and distribution of endophytic fungi within seed and plants of pasture grasses have been known since the last century (McLennan 1920, for a review) but only rarely have these endophytic fungi been identified or have sporulating structures or in vitro colony characteristics been described or illustrated. Sampson (1933, 1937) isolated two endophytes from perennial ryegrass (Lolium perenne L.); one remained sterile and the second formed penicillately branched conidiophores. She (Sampson 1933) also found Epichloe typhina (Pers. : Fr.) Tul. growing endophytically within Chewings fescue [Festuca rubra L. sensu lato]. Neill (1940) isolated an endophyte from ryegrass and published a photograph of the sterile colony growing on agar. He later (Neill 1941) described microconidia and macroconidia from cultures of this endophyte. Neill (1941) isolated a second endophyte, from tall fescue (Festuca arundinacea Schreber), that he identified as Epichloe typhina. Bacon et al. (1977) also found E. typhina growing endophytically within tall fescue in the U.S.A. Morgan-Jones & Gams (1982) described Acremonium coenophialum Morgan-Jones & Gams as an endophyte of tall fescue. Balansia epichloe (Wouse) Diehl has been found to be an endophyte of tall fescue, Bermuda grass [Cynodon dactylon (L.) Pers.], Sporobolus poiretii (Roem. & Schult.) Hitch., Andropogon sp. and Eragrostis sp. (Bacon

EXHIBIT C: PAGE 1 OF 9

et al. 1975, Porter et al. 1975) and an unidentified Balansia sp. has also been reported from Bermuda grass (Porter et al. 1975).

This paper reports five endophytes from pasture grasses in New Zealand, two from Lolium perenne, two from Festuca arundinacea and one from F. rubra. The systematic relationships of these endophytes are discussed.

MATERIALS AND METHODS

Detection of endophytes in plants and seeds.

A leaf sheath was removed from the plant, the adaxial epidermis was cut with a scalpel and then stripped from the sheath with forceps. The epidermal tissue was mounted in 0.1% cotton blue in lacto-phenol and the slide warmed for a few seconds over a flame to accelerate staining of mycelium. The slide was examined under low power of a microscope for the presence of mycelium.

The presence of viable endophyte in seed was determined indirectly by germinating fifty seeds in sterilized soil, and then examining the leaf sheaths 3-6 weeks later for mycelium.

Isolation of endophytes.

Leaf sheath tissue was surface sterilized by dipping in 70% ethanol for 5 seconds, then shaking in a solution of 1% sodium hypochlorite (0.5% available chlorine) for 5 minutes. Seed was surface sterilized by soaking in 50% sulfuric acid for 20 minutes followed by rinsing in sterile water, soaking in 10% sodium hypochlorite for 20 minutes and rinsing again in sterile water.

A method based on that described by Neill (1940) was used initially for isolations. A single piece of surface sterilized tissue, approximately 2 cm long, from the leaf sheath and adjoining proximal end of the leaf, was placed in a test tube containing 10 ml potato dextrose broth with 1 ppm penicillin and 100 ppm streptomycin. Surface sterilized seeds were also placed in test tubes of the same broth, one seed in each tube. The test tubes were incubated at 18-22°C either in darkness or in diffused daylight for two days within which time mycelium was observed on the excised leaf tissue and on the emergent seeds by transferring seeds. After preliminary assessment of this method it was found that fungi could be isolated more easily if surface sterilized leaf tissue or seed, or mycelium from sterile hyphae of A. typhina stromata on cowdung tissue was plated below the surface of potato dextrose agar (PDA) in petri dishes incubated at 18-22°C.

Cultural characteristics of fungi isolated.

Ten isolates of the endophytes, given below, are based on cultures grown on oatmeal dextrose agar (OMD), PDA and oatmeal agar (OA). Colony diameters were measured as described in Table 1. All measurements were made from material mounted in lacto-lactic acid. Prepared microscope slides and dried cultures of all endophytes have been deposited in the herbarium of the Plant Diseases Division, Auckland (PDD). Selected living cultures have been deposited in the Plant Diseases Division Culture Collection (PDDCC), the American Type Culture Collection and the Centraalbureau voor Schimmelcultures.

Representative cultures of all the endophytes described have been inoculated into their appropriate hosts, observed as endophytic mycelium, and successfully isolated from inoculated plants as cultures indistinguishable from those originally inoculated. These results will be described in a forthcoming publication.

DESCRIPTIONS OF THE SPECIES

Five endophytic fungi were isolated from three grasses. They were identified as Acremonium loliae n.sp. and a Gliocladium-like fungus from perennial ryegrass; Acremonium coenophialum and a Phialophora-like fungus from tall fescue; and Epichloe cf. typhina (Persoon) Tulasne from Chewings fescue.

Description of mycelium in plants.

All five endophytic fungi have septate, intercellular hyphae. Mycelium of A. loliae, A. coenophialum and Epichloe cf. typhina runs parallel to the leaf axis and is infrequently branched. A. coenophialum differs from the other two fungi in that the mycelium is slightly more convoluted.

Mycelium of a Gliocladium-like endophyte in ryegrass is strongly branched and there is a greater bulk of mycelium present than with A. loliae. When ryegrass is infected with both of these fungi it is sometimes difficult to identify the mycelium of A. loliae because of the profusion of Gliocladium-like mycelium.

Although A. coenophialum and a Phialophora-like endophyte of tall fescue are often present in the same plant, mycelium of the latter fungus is frequently branched and readily distinguished from that of A. coenophialum.

In mature plant tissue the constrictions at the septa in hyphae of all five fungi are more distinct. In the Gliocladium-like and Phialophora-like fungi these constrictions are so pronounced that the mycelium appears bead-like.

I. The Lolium endophytes

1. Acremonium loliae Latch, Christensen & Samuels, sp. nov. Fig. 1 E.

Coloniae fere lente crescunt, albae. Odor nullus. Hyphae vegetativae 1-2 µm latae. Phialides singulae ex hyphis aeriis oriundae, septo basilari adsunt, acrifeatae, (8.0-)13.5-65.1(-110.0) µm longae, e 1.5-2.0 µm plaulatim ad 1.0-1.5 µm angustatae. Conidia solitaria, saepe transversaliter affixa, ellipsoidea vel reniformia, hyalina, (3.0-)4.7-7.3(-13.0) x (1.5-)1.9-2.5(-3.0) µm.

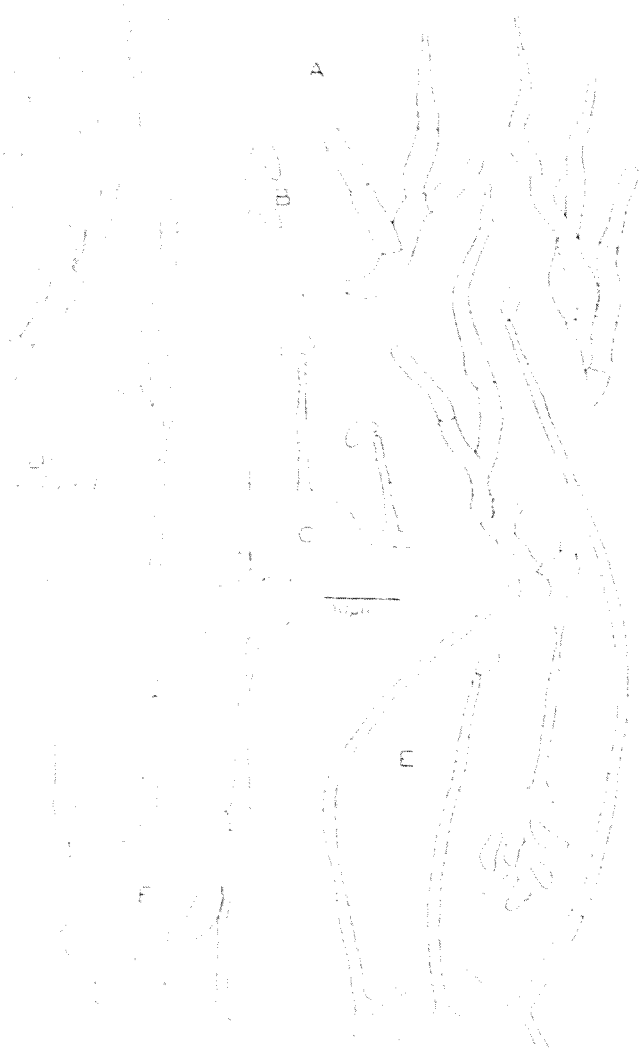

Fig. 1. A. Epichloe cf. typhina (PDD 40914) conidiophores and aerial hyphae from Festuca rubra. B. Conidia from culture (PDD 40914). C. Conidiophores from CMD, the two on the left drawn from culture (T-6), the one on the right with transversely oriented conidia drawn directly from culture (T-6). D. Conidia from culture (T-6). E. Acremonium loliae conidiophores and conidia from MD (H 103). F. Acremonium coenophialum conidiophores and conidia; three conidiophores on the right drawn directly from culture.

In culturis ex Lolii perenni, Castlepoint, North Island, New Zealand, G.C.M. Latch et M.J. Christensen R 26, 8 Sep 1981. (PDD 44917).

Colonies grown for five weeks on PDA tan, hemispherical, convoluted, aerial mycelium lacking, colony sterile (Table 1). Colonies grown on CMD and OA white, flat, waxy, with or without short, white aerial hyphae; surface of some colonies on OA tan, concolorous with the medium, flat or slightly raised, waxy and convoluted, aerial mycelium lacking. Aerial mycelium, when present, consisting of scattered, erect hyphae, less frequently scant and loosely cottony; hyphae 1.5-2.0(-2.5) µm wide. Conidiophores forming abundantly on CMD at 4° and 10°C, less abundantly at higher temperatures; only from aerial hyphae, hyaline, unbranched, straight, smooth, aseptate, tapering gently from 1.5-2.0 µm wide at base to 1.0-1.5 µm wide at tip, (8.0-)13.5-65.0(-110) µm long, lacking a basal septum or, rarely, 1-2-septate, wall not thickened, lacking a septum in the subtending hypha near the base of the conidiophore; with a single conidiogenous cell. Tip of conidiogenous cell lacking periclinal thickening and not flared, either bearing a developing conidium or closed, conidia produced singly, often transversely positioned on the tip of the conidiophore. Conidia ellipsoid to reniform, ends rounded, lacking a basal abscission scar, (4.0-)4.7-7.3(-13.0) x (1.5-)1.9-2.5(-3.0) µm [N = 207, M_l = 6.0, SD = 1.3 µm; M_w = 2.2, SD = 0.3 µm] unicellular, hyaline.

TABLE 1. Growth of Epichloe cf. typhina, Acremonium loliae and A. coenophialum measured after 3 and 5 weeks in darkness.

	Colony diameter[1] (mm, 3 weeks/5 weeks)					
Temperature (°C)	0	4	10	15	20	25
Epichloe cf. typhina						
OA	+/+[2]	10/20	9/15	10/25	29/40	28/45
PDA	+/+	12/17	10/15	8/25	32/47	32/50
CMD	+/+	12/20	6/17	15/27	35/45	32/45
Acremonium loliae						
OA	0[3]/0	+/8	+/7	9/7	8/15	8/15
PDA	0/+	+/9	+/8	+/9	+/8	+/9
CMD	0/+	+/7	+/7	7/8	9/10	9/11
Acremonium coenophialum						
OA	+/+	+/+	+/+	+/7	8/15	8/17
PDA	0/0	+/8	+/8	+/7	10/18	8/18
CMD	+/+	+/6	+/6	+/8	9/17	9/17

[1]Diameter includes 3 mm diam. plug of inoculum (CMD).

[2]Erect hyphae evident on the inoculum.

[3]No growth evident on the inoculum.

holotype: Isolated by G.C.M. Latch & M.J. Christensen on 26, 5 Sep 1981, from leaf tissue of Lolium perenne collected at Castlepoint (PDD 44517, PDDCC 8360).

Isolated from seeds and leaf sheaths, but not from culms, of 450 plants of Lolium perenne collected in the North Island and from Canterbury. The following isolates are considered to be typical: R 100 (PDD 44528, PDDCC 8361), R 150 (PDD 44490, PDDCC 8362), R 103 (PDD 44529, PDDCC 8363).

2. Gliocladium-like isolates Fig. 2 A-C.

Colonies grown five weeks on PDA forming a cottony, white, hemispherical button over the inoculum, colony otherwise flat; aerial mycelium fluffy on PDA but lacking on CMD and OA; yellow pigment evident in colony reverse of all media but most pronounced on PDA. Hyphae 1.5-2.0 µm wide, septate, branched, often forming loops on surface of agar and in aerial hyphae. Indehiscent, Chrysosporium-like cells, 3-6 x 1.5-2.0 µm arising laterally and terminally on immersed hyphae. Colonies generally sterile, occasionally a few Gliocladium-like conidiophores arising as lateral branches of aerial hyphae. Conidiophores well differentiated, mononematous, straight, smooth, colorless to pale yellow, 10-15 x 3-5 µm, branching apically to form a penicillus of phialides. Phialides cylindrical, 6-9 x 3.0-3.5 µm, held singly or in whorls of up to 3 directly on conidiophore or on metulae ca. 10 µm long; metulae, hyaline, smooth, tip with periclinal thickening, not flared, conidia held in heads of colorless slime. Conidia oblong to ellipsoid, with a round to flat base, (3.5-)3.6-4.0(-6.0) x (1.5-)1.8-2.3(-2.5) µm [N = 44, M_l = 3.8, SD = 0.7 µm, M_w = 2.0, SD = 0.3 µm].

Isolated from seeds, roots and leaf sheaths of 87 plants of Lolium perenne collected in the central region of the North Island and from Canterbury. The following isolates are considered to be typical: R 72 (PDD 44526, PDDCC 8497), R 161 (PDD 44531, PDDCC 8498), R 169 (PDD 44532, PDDCC 8499).

TABLE 1. Growth of Gliocladium-like ryegrass endophyte after 3 and 5 weeks at 5 temperatures in darkness.

	Colony diameter[1] (mm, 3 weeks/5 weeks)					
Temperature (°C)	0	5	10	15	20	25
Medium:						
OA	+[2]/+	22/32	30/65	23/52	7/18	7/12
PDA	+/+	24/42	24/52	35/47	7/21	7/13
CMD	+/+	20/35	20/37	22/37	9/18	5/10

[1] Diameter includes 5 mm diam. plug of inoculum (CMD).

[2] + = Hyphae evident on inoculum.

II. The Festuca endophytes

1. Epichloe cf. typhina (Persoon : Fr.) Tulasne, Sel. Fung. Carp. 3: 24. 1865. Fig. 1 A-D.

Anamorph: Sphacelia typhina Saccardo, Michelia 2: 297. 1881.

= Acremonium typhinum Morgan-Jones & Gams, Mycotaxon 15: 315. 1982.

Colonies grown five weeks on PDA, CMD and OA with short, white to ivory, aerial hyphae, or no aerial mycelium (diameters given in Table 1). Colonies grown on OA waxy with very little or no aerial mycelium, tending to become wrinkled with age. Colony reverse on PDA with light brown coloration in the center; colony reverse on CMD white. Hyphae 1-2 µm wide. Conidiophores forming abundantly in all media from aerial hyphae and from surface of colony, hyaline, unbranched, straight, smooth, aseptate. Tapering gently from 1.0-1.5(-2.0) µm wide at base to 0.5-1.0 µm wide at tip, (8.0-)11.6-19.7(-20.0) µm long, lacking a basal septum, with or without a septum in the subtending hypha near the base of the conidiophore, wall of base of conidiophore sometimes thickened; with a single conidiogenous cell. Tip of conidiogenous cell lacking periclinal thickening and not flared, either bearing a developing conidium or closed, conidia produced singly, often situated transversely at the tip of the conidiophore. Conidia ellipsoid to asymmetric and shaped like an orange fruit segment, with one side flattened and one side curved, lacking an obvious basal abscission scar. (3.5-)4.3-5.7 (-9.0) x (1.5-)1.9-2.7(-4.0) µm [N = 231, M_l = 5.0, SD = 0.8 µm, M_w = 2.3, SD = 0.4 µm], unicellular, hyaline.

Isolated from mycelium of young Epichloe stroma on six plants and from leaf sheaths of ten symptomless plants of Festuca rubra from the central region of the North Island. The following isolates are considered to be typical: T 2 (PDD 44550, PDDCC 8509), T 4 (PDD 44547, PDDCC 8500), T 5 (PDD 44548, PDDCC 8501), T 6 (PDD 44549, PDDCC 8502).

NOTE. Sampson & Western (1954) and Western & Cavett (1959) described the disease cycle of E. typhina and noted that conidia are formed on young stromata but they did not give morphological details of this anamorph. Booth (1979) reported that young stromata of E. typhina are covered with Acremonium-like conidiophores but he neither described nor illustrated details of the conidiophores. Six of our New Zealand isolates of Epichloe cf. typhina were derived from mycelium and conidia of the young stroma that was found on culms of Chewings fescue. There were no obvious signs of perithecial formation on these stromata. Perithecia of E. typhina have not been reported from New Zealand and the fungus has only recently been noticed in the country from F. glauca Lam. (Hedley & Braithwaite 1978).

The culms of Chewings fescue were girdled by a 1.5-3.0 cm long, white stroma that was composed of tightly compacted, 3 µm wide hyphae that were arranged

perpendicularly to the surface of the stroma. Conidiophores (Fig. 1 A) were unbranched, septate, 22-65 µm long and formed in a compact layer over the surface of the stroma, each as an extension of underlying hyphae. A single conidiogenous cell constituted the terminal 17-26 (-18) µm of each conidiophore. The tip of the conidiogenous cell lacked obvious periclinal thickening and was flared. Simple conidiophores, as found in culture (Fig. 1 C), were not observed in our material. Conidia were as found in culture (Figs. 1 A,D). Sterile, 3-4 µm wide hyphal hairs arose from the base of conidiophores or arose separately from stromal hyphae; they were septate, unbranched, smooth with round tips and attained the same length as the conidiophores. These observations broadly agree with those of Vincens (1917, fig. 65) for *E. typhina*.

2. *Acremonium coenophialum* Morgan-Jones & Gams, Mycotaxon 15: 313. 1982. Fig. 1 F.

Colonies grown five weeks on PDA, CMD and OA flat, aerial mycelium lacking to short, white, surface of colony appearing felty (colony diameters given in Table 1). Colonies grown on OA waxy and wrinkled with little or no aerial mycelium with age. Aerial hyphae on CMD branched, [illegible] µm wide. Conidiophores forming abundantly on all media from aerial hyphae and from surface of colony, hyaline, unbranched, straight, smooth, aseptate, or, rarely [illegible] septate near base, tapering gently from 1.5-2.0 µm wide at base to [illegible] µm wide at tip, 12-56(-155) µm, some at the ends of hyphae and then indeterminate in length, [illegible] a basal septum, with or without a septum in the [illegible] hyphae near the base of the conidiophore, wall [illegible] width as hyphal wall; with a single conidiogenous [illegible]. Tip of conidiophore lacking periclinal thickening and not flared, either bearing a developing conidium or [illegible]. Conidia produced singly, often transversely [illegible] on the tip of the conidiophore. Conidia [illegible] to falcate, tip round to subacute, base round or with a protuberant, flat abscission scar, (7.0-)9.5-12.8 (-[illegible]) x (2.0-)2.4-3.6(-[illegible]) µm [N = 300, M_l = 11.0, SD = [illegible] µm, M_w = 3.0, SD = 0.6 µm], unicellular, hyaline.

Isolated from seeds and leaf sheaths, but not roots, [illegible] plants of *Festuca arundinacea* from the central region of the North Island; three isolates from the U.S.A. were [illegible]. The following isolates are considered to be [illegible]: F 21 (PDD [illegible], PDDCC [illegible]), F 27 (PDD [illegible], PDDCC [illegible]), F 3? (U.S.A.) (PDD [illegible], PDDCC [illegible]).

3. *Phialophora*-like isolates Fig. 2 D.

Colonies grown three weeks on PDA, CMD and OA raised [illegible] the original inoculum and aerial mycelium densely [illegible], colony otherwise flat with scant aerial mycelium; aerial mycelium white to off white to light tan or [illegible] brownish tan with [illegible] brown to grey coloration in center [illegible] reverse; colonies on OA with slightly spreading [illegible] pigment. Sometimes yellow drops of liquid forming [illegible] aerial mycelium of all media and sometimes with yellow [illegible] in reverse of colonies grown on OA. Hyphae

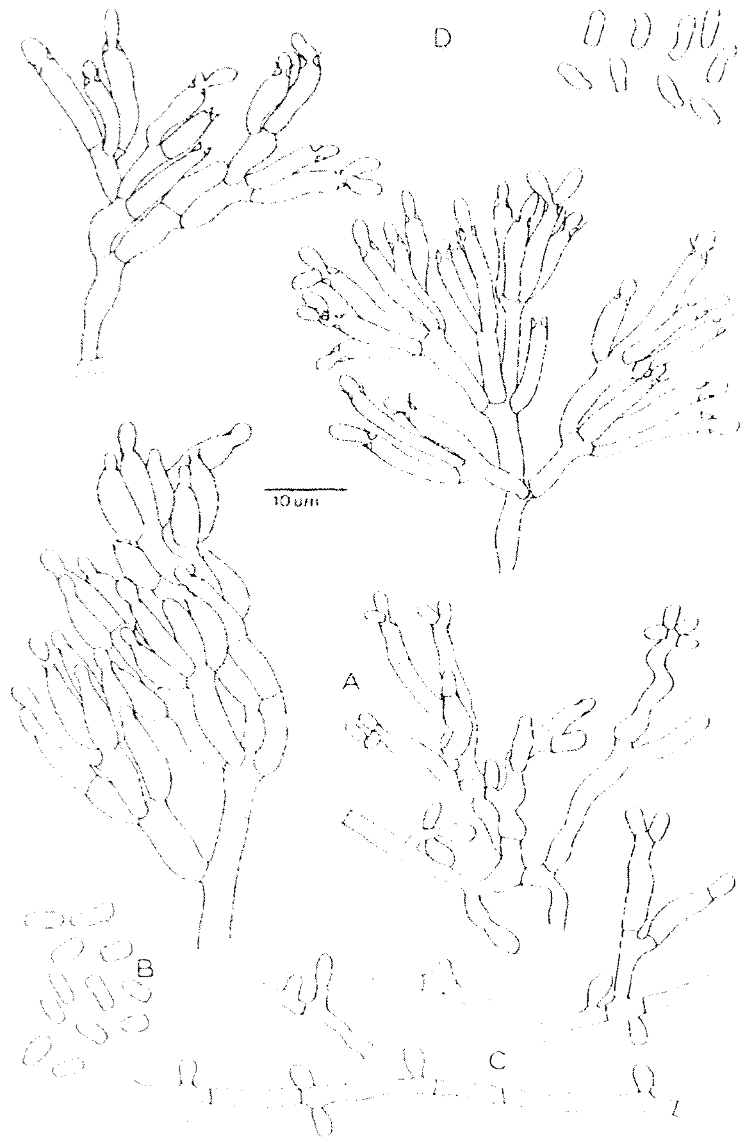

Fig. 2. *Gliocladium*-like endophyte of *Lolium perenne*. A. Conidiophores (R 162 on right, R 169 on left). B. Conidia (R 162). C. Indehiscent hyphal protuberances. D. *Phialophora*-like endophyte of *Festuca arundinacea*.

...wide, septate, branching. Conidial production more profuse on OA than on CMD. Conidiophores arising from surface of colony and in aerial mycelium, tending to form ... fascicles, hyaline, smooth, 25-70 µm long, penicillately branched at the tip, branches 8-10 µm long, each bearing a penicillus of phialides. Phialides cylindrical, (8-)10-15 (-18) x 2-3 µm, hyaline, smooth, tip with pronounced periclinal thickening or periclinal thickening not evident, often widely flared; often with one or two conidia attached to the phialide tip within the collarette, conidia held in heads of colorless slime. Conidia oblong to ellipsoid to clavate with a flat base, 1 µm wide (3.0-)3.8-5.4(-7.0) x (1.5-)1.7-2.2(-2.5) µm [N = 67, M_1 = 4.6, SD = 0.9 µm, M_w = 1.9, SD = 0.2 µm], unicellular, hyaline.

Isolated from seeds, roots, and leaf sheaths of 58 plants of Festuca arundinacea from the central region of the North Island. The following isolates are considered to be typical: F 5 (PDD 44451, PDDCC 8505), F 17 (PDD 44452, PDDCC 8506), F 20 (PDD 44462, PDDCC 8507).

TABLE 1. Growth of Phialophora-like fescue endophyte after 3 weeks at different ...

Isolate	Colony diameter[1] (mm) 10°	15°	20°	25°
MEA				
F 5A	12	15	6	+[2]
F 5B	15	15	6	+
F 5C	7	12	5	+

[1] Excludes inoculum plug of inoculum (CMD).

[2] + = slight growth evident on inoculum.

DISCUSSION

...

Several recent papers (Fletcher & Harvey 1981, ... et al. 1981, Latch & Christensen 1982, Finnan et al. 1982) have discussed the relationship of the toxic ... Ryegrass Staggers in cattle and sheep to "Neill's ... Lolium ... endophyte." In some of the recent papers ... have discussed "Lolium endophyte" has the fungus under ... from ... effort of named and evidence has not been ... that the fungus was actually the same fungus that ... studied. The identity of the fungal ... in these recent studies is therefore in doubt.

*But is "Neill's Lolium endophyte?" We will never ... for answer to this question because Neill left no ... et al. Neill (1940) first described a non-... and phiale that had waxy, convoluted colonies on ... Later (Neill 1941) he described microconidia and ... that had been incubated for five to eight weeks ... and soil). These conidia were similar

to but distinct from those formed by Gloeotinia granigena (Quelet) Schumacher [= G. temulenta (Prill. & Delacroix) Wilson, Noble & Gray], the cause of blind seed in ryegrass (Neill & Hyde 1939). The overall morphology of the microconidial and macroconidial sporodochia are as described for G. granigena and it is possible that Neill was observing a species of Gloeotinia.

Neill (1940) obtained his first endophyte from "over 90 percent of infected seedlings from three lines of certified perennial." The cultures that he described and the high incidence of this cultural type in ryegrass, strongly suggest Acremonium loliae as described above. If Neill was isolating A. loliae, the subsequently observed sporulation could have been of a contaminant. We followed his techniques for isolation of endophytes and occasionally isolated G. granigena and A. loliae from the same seedling. We did not encounter any fungus with the combination of cultural characters (Neill 1940) and conidia (Neill 1941) of Neill's "first endophyte."

Sampson (1933) reported endophytic mycelium in seed, leaves, stems and tiller buds of darnel (Lolium temulentum L.) and ryegrass and she later (Sampson 1937) published cultural details of the fungus that she isolated from ryegrass and referred to as the "first endophyte." Although she did not observe sporulation for this species, the cultures that she described and illustrated (Sampson 1937, Pl. IX, fig. 12) are similar to those produced by A. loliae in our study and we believe that Sampson's "first endophyte" was A. loliae. That neither Sampson (1937) nor Neill (1941) observed conidia in their isolates might be explained by the fact that A. loliae sporulates poorly at temperatures above 15°. Neither Sampson nor Neill were able to isolate the darnel endophyte.

In our work (unpublished), the fungus most commonly isolated from ryegrass was Acremonium loliae, and this species was consistently associated with outbreaks of Ryegrass Staggers. Work is continuing on the relationship of this fungus to this toxic syndrome.

Another species of Acremonium has been linked to animal toxicity. A. coenophialum was originally isolated as an endophyte of tall fescue in Alabama, U.S.A. (Morgan-Jones & Gams 1982) and Morgan-Jones & Gams (1982) briefly remarked on the association of this species with "fescue toxicity" in cattle. Bacon et al. (1977) reported the association of Epichloe typhina with this toxic syndrome but the conidia that they described were larger than those reported by Brefeld & Tavel (1891) and Morgan-Jones & Gams (1982) for E. typhina. The distinctive conidia and wrinkled colonies that Bacon et al. (1977) described resemble those of A. coenophialum. Three of our isolates of A. coenophialum originated in Kentucky, U.S.A. The U.S.A. and New Zealand isolates agree in every respect with the original description of A. coenophialum. Neill (1941) described and illustrated an Acremonium-like anamorph that he found to be endophytic within apparently healthy tall

fescue plants in New Zealand. He referred to his fungus as E. typhina. Morgan-Jones & Gams (1982) suggested that this fungus could have been A. coenophialum and we agree.

A. coenophialum, A. loliae, and the Acremonium-like anamorph of Epichloe typhina (sensu Brefeld & Tavel 1891) are strikingly similar in morphology and cultural features. These three anamorphs form a distinctive and homogeneous group for the following reasons: 1). the base of the conidiogenous cell consistently lacks a septum; 2). there is no periclinal thickening at the tip of the conidiogenous cell, which is either closed or bears a developing conidium; and 3). usually only one conidium, and at most two, are found at the tip of the conidiogenous cell, and that conidium is often transversely situated. These species vary from typical Acremonium (Gams 1971, Samuels 1976) wherein conidiophores are basally septate, phialides have periclinal apical thickening, and conidia are held in a conspicuous drop of liquid.

Morgan-Jones & Gams (1982) described the anamorph of E. typhina as Acremonium typhinum Morgan-Jones & Gams. They did not illustrate periclinal thickening at the conidiogenous locus. Although they did not state whether conidia are borne in a drop of liquid, they did describe transverse positioning of a single conidium at the tip of the conidiogenous cell. Brefeld & Tavel (1891) isolated ascospores of E. typhina into pure culture and illustrated (vol. V, Taps. IV, VI) transverse positioning of a single conidium on the anamorph, but conidiophores differed from those described for A. typhinum in lacking a basal septum. Although it was not stated whether the type culture of A. typhinum was isolated from fungal fructifications or from plant tissue, the isolate of the species was derived from ascospores. These different anamorphs that are the exclusive or two taxa whose perithecia are morphologically similar. But New Zealand isolates are derived from infection of tissue of plants of Chewings fescue that lacked stromata of E. typhina and from ascertium of young Epichloe stromata of other plants, and agree with Brefeld & Tavel's (1891) illustrations.

Saccardo (1881) described the anamorph of E. typhina as Sphacelia typhina Sacc. [as '(Pers.) Sacc.'] and this name has generally been accepted for the anamorph of E. typhina (Subramanian 1971, Ahx 1981). Morgan-Jones & Gams (1982) were unable to find the described fungus on either of the isotype specimens of Sphacelia typhina in Saccardo's herbarium (PAD); they did not examine other naturally formed anamorphs of E. typhina and they did not discuss whether Sphacelia Lev. is the appropriate genus for this anamorph.

Sphacelia segetum Lev., the type species of Sphacelia, is the anamorph of Claviceps purpurea (Fr.) Tul., another graminicolous member of the Clavicipitales. In this anamorph Acremonium-like conidiophores form in a compact palisade on the surface of the ergot sclerotium (Tulasne & C. Tulasne (v. 3, Fig. 328; Barnett & Hunter 1972). The

anamorphs of Epichloe typhina and Claviceps purpurea have the same basic morphology and therefore seem to be congeneric. The form of these anamorphs in nature is comparable to the Ephelis anamorph of a third graminicolous member of the Clavicipitales, Myriogenospora atramentosa (Berk. & Curt. in Bork.) Diehl (Rykard et al. 1982). Anamorphs of graminicolous members of the Clavicipitales thus have complex conidiomata that conform to the contour of the presexual stroma. The same is observed in other non-clavicipitaceous ascomycetes. Perithecia of Nectria cinnabarina (Tode : Fries) Fries form on the surface of the sporodochium of Tubercularia vulgaris Tode after the cessation of conidial production (personal observation). When Epichloe typhina or N. cinnabarina are grown in pure culture, however, conidiophores do not aggregate into stromata, and in Myriogenospora atramentosa and other members of the tribe Balansiae (Diehl 1930) conidiogenous hyphal aggregates and non-aggregated conidiophores form in agar culture. Separate generic names are not given to the cultural expressions of the Balansiae or Nectria and we do not believe that the cultural expression of E. typhina warrants a separate name in Acremonium. Therefore, we reject Acremonium typhinum as the appropriate name for the anamorph of Epichloe typhina. In the absence of a critical review of taxonomy of the anamorph genera attributed to the graminicolous Clavicipitales, we prefer to refer the anamorph of E. typhina to Sphacelia.

That conidia and conidiophores of Acremonium coenophialum and A. loliae are so strikingly similar to those of the Acremonium-like conidiophores formed in cultures of E. cf. typhina suggests that there may be three species of Epichloe present in New Zealand pasture grasses but that they rarely or never form visible fructifications. We accept these species in Acremonium sect. Albo-lanosa Morgan-Jones & Gams (1982) on the basis of morphology of the conidiophore alone and in the belief that they are clavicipitaceous anamorphs and probably unrelated to other Acremonium species. Because periclinal thickening is not seen in the tip of the conidiogenous cell and because only one conidium is borne on each conidiogenous cell, it is possible that conidiogenesis in these three species is holoblastic, thus separating them further from other Acremonium species. Whether A. coenophialum and A. loliae should be placed in a separate genus should be considered by specialists in hyphomycete taxonomy. Conidiogenesis in Myriogenospora atramentosa is holoblastic but the conidiogenous locus proliferates sympodially after the formation of each conidium (Rykard et al. 1982).

The Gliocladium and Phialophora endophytes.

The Gliocladium-like and Phialophora-like endophytes described in this paper are very generalized in morphology. Their generic names reflect that morphology without implying phylogenetic relationship to other species of the genera. We refer to the ryegrass endophyte as being

"Gliocladium-like" because of the light pigmentation in the [illegible], and the penicillate arrangement of phialides, because the tips of the phialide are neither flared nor cupulate or tubular, and because the conidia are unicellular, colorless and held in colorless slime. The [illegible] endophyte is referred to as being "Phialophora-like" because of the brown pigmentation of some colonies, because phialides often had widely flaring apical collarettes, and because conidia are unicellular and held in slime. This endophyte bears at least some morphological resemblance to branched species of Phialophora Medlar (Schol-Schwarz 1970). We do not know whether either of these endophytes forms complex conidiomata in nature.

The Gliocladium-like and Phialophora-like endophytes described above bear morphological similarities to endophytes previously found in Lolium species by Sampson ([illegible]) and referred to as the "second endophyte." Sampson ([illegible], figs. 10-15) described and illustrated a seed-borne endophyte of Lolium perenne that was penicillately branched and that formed conspicuous hyphal loops in the aerial mycelium. The mycelium on oatmeal agar was cottony and white [illegible] in color and a "chrome-yellow" pigment formed in the medium. Conidia formed abundantly, were [illegible], measured 2.0-2.5 x 1.5 µm, and were [illegible]. Details of conidiogenesis were not [illegible]. These conidia are smaller than those of our Gliocladium-like and Phialophora-like endophytes. Sampson's "second endophyte" and our Gliocladium-like endophyte appear to differ only in conidial size.

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FINNISH RECORDS OF DISCOMYCETES: A NEW SPECIES IN THE MONOTYPIC GENUS DESMAZIERELLA (PEZIZALES)

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SUMMARY

A new species, *Desmazierella picicola*, occurring on needles of *Picea abies* is described. In many characters it is related to *D. acicola* Lib., but differs especially in the gigantic and fusoid spores with cyanophilous ridges. The genus *Desmazierella* is considered to belong to the family Sarcoscyphaceae, tribe Pithyeae.

Studies on collections of the University of Turku Herbarium (TUR) revealed a discomycetous specimen with an unusual combination of characters. The collection was on a largely decomposed spruce needle dating from 1965. Only two apothecia were observed, and type material is therefore scanty. However, original drawings and permanently mounted material on slides are enclosed in the type collection. The type locality has since been visited frequently but without result.

Desmazierella picicola Huhtinen & Mäkinen, spec. nov.

Apothecia 1 mm lata, breviter stipitata, ad maturitatem cupulata. Hymenium griseum, brunneosetosum; apothecia exteriori parte brunnea, dense pilosa. Excipulum externum hyalinum vel brunneum, textura prismatica vel textura angulari, bistratus vel tristratus, cellulis 10-15 X 6-10 µm. Excipulum medullarium hyalinum, textura intricata, hyphis ad 5-10 µm latis, ramosis, non gelatinosis. Pili usque ad 1000 µm longi, erecti et setosi vel flexuosi, multiseptati, superficiali, parietibus usque ad 4 µm crassis, brunneis vel atrobrunneis, irregulariter granulosis; brevissimi pili abundantes, brunnei, granulati, 10-20 X 5-9 µm. Asci 300 X 12 µm, tetraspori, cylindracei, parietibus crassis, non amyloidei, basi irregulariter bifurcata, operculo apicali, crassa. Ascosporae 48-57 X